The Dissertation Committee for Alan Bayard Watts Certifies that this is the approved version of the following dissertation:

Pulmonary Delivery of Tacrolimus for Lung Transplant and Asthma Therapy

Committee:

Robert O. Williams III, Supervisor

James W. McGinity

Keith P. Johnston

Jay I. Peters

Robert L. Talbert

Pulmonary Delivery of Tacrolimus for Lung Transplant and Asthma Therapy

by

Alan Bayard Watts, B.S.

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Dedication

To my family, and in loving memory of Sarah E. Harris

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Pulmonary Delivery of Tacrolimus for Lung Transplant and Asthma Therapy

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Since the discovery of cyclosporine in 1971, calcineurin inhibitors have played a critical role in the therapeutic suppression of the immune response. Patients receiving solid organ transplants rely heavily on these medications to prevent the acute and chronic rejection of allografted tissue. Introduction of tacrolimus, the most frequently prescribed calcineurin inhibitor, has lead to improved clinical outcomes for organ transplant recipients; however, little improvement has been noted in lung transplantation. Difficulties with current oral dosing regimens for lung transplant patients stem primarily from drug systemic toxicity, heightened risk of invasive infection, and erratic oral bioavailability. We have proposed that pulmonary delivery of a tacrolimus formulation with improved solubility can provide high lung concentrations, while limiting corresponding systemic levels associated with toxicity.

Chapter 2 investigates the pulmonary administration of tacrolimus dispersion for nebulization to lung transplanted rats. Resulting lung and blood levels were determined

by appropriate bioanalytical methods. Limited systemic absorption was seen after pulmonary delivery, resulting in a 50 to 1 lung to blood concentration ratio.

A 28 day safety and stability evaluation of tacrolimus dispersion for nebulization was conducted in Chapter 3. Results showed no signs of toxicity in Sprague Dawley rats and proved the stability of tacrolimus powder for dispersion for 3 months.

For cases of severe asthma, immunosuppression is also necessary to restore normal lungs function and is typically treated with corticosteroids. Corticosteroids, however, are well know for their untoward side effects and can prove ineffective in severe asthmatics that have developed corticosteroid resistance. Chapter 4 investigates the use of tacrolimus dispersion for nebulization for prophylactic treatment of asthma. Efficacy was determined in an asthma-induced animal model by quantification of inflammatory cells and signaling chemicals.

In Chapter 5, tacrolimus powder for inhalation is investigated in a novel dry powder inhalation platform. Respirable particles are produced when bulk particles (500 μm) comprising a matrix of drug/excipient are sheared apart by a marketed inhalation device to produce particles of the appropriate geometric diameter (50 μm). Biocompatible material with brittle properties were found to produce fine particle fractions (FPF) up to 70.3% and total emitted doses (TED) higher than 95%.

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Chapter 1: Recent Developments in Drug Delivery to Prolong Allograft Survival in Lung Transplant Patients

1.1 INTRODUCTION

Of the major forms of solid organ transplantation (kidney, liver, heart, and lung), lung transplantation has proven to be the most difficult in providing effective immunosuppression, while maintaining the body's ability to protect against invasive infection. Further risks of infection are added in lung transplanted patients by the disruption of the mucociliary escalator and cough reflex (1). While consistently improving, survival of lung transplant patients lags behind other forms of transplantation with an approximate 50% survival rate after 5 years. Chronic allograft rejection caused by bronchiolitis obliterans syndrome (BOS) effects 50 to 60% of 5-year post-operative patients and is a major hurdle in improving quality of life and patient survival (2). To prevent chronic rejection, maintenance immunosuppressive therapies consisting of a three drug regimen (calcineurin inhibitor, antimetabolite, and corticosteroid) are typically administered (3). Calcineurin inhibitors hinder T-cell proliferation, and the subsequent immune response, by complexing with immunophillins and binding to calcineurin, resulting in the inhibition of cytokine transcription. Antimetabolites, such as azathioprine and mycophenolic acid, reduce expansion of T and B cells by blocking purine synthesis (4). Corticosteroids have a broad mechanism of action which is not completely understood (5); however, they are effective inhibitors of T-cell and macrophage cytokine production. Withdrawal from prolonged corticosteroid therapy can be particularly troublesome in lung transplant patients due to dangerous side-effects including

hypertension, increased cholesterol, and osteoporosis. (6, 7). A new method of therapy has been developed in poly- and monoclonal antibodies. Monoclonal antibodies such as basiliximab and daclizumab bind specifically to IL-2R surface protein, inhibiting signaling for further T cell expansion. A schematic depicting the cycle of CD4 T cell activation with sites of immunosuppressive action is shown in Figure 1.1. Development of new immunosuppressive drugs (i.e. daclizumab, basiliximab, everolimus, FK788) has further raised expectations of successful immunosuppressive therapy. The purpose of this review paper is to summarize how advances in drug delivery may lead to improved clinical outcomes specifically relating to lung transplants. The majority of research done in this area investigates improved delivery of calcineurin inhibitors cyclosporine and tacrolimus.

In this review, innovative drug delivery with the potential to improve survival rate in lung transplanted patients is discussed. Many of these delivery methods are currently used extensively in transplantation therapy and also may be applied to lung transplant recipients, while other novel methods are specifically intended for rejection prevention specifically in transplanted lung patients.

1.2 DELIVERY SYSTEMS INTENDED FOR THE ORAL ROUTE OF ADMINISTRATION

Consistent oral immunosuppressive regimens are common in the clinical setting as well as during long term maintenance therapies. Self administration and patient compliance make the oral delivery route attractive; however, toxic side-effects, variations in clearance (8) and bioavailability of some poorly soluble immunosuppressive drugs present obstacles in maintaining therapeutic drug levels. Many novel formulation approaches have been developed for lung (9, 10), as well as other solid organ transplant recipients, to prevent acute and chronic allograft rejection.

1.2.1 Cyclosporine

In all solid organ transplantation, including lung transplantation, oral cyclosporine has been frequently used to block T cell proliferation; although, due to its improved potency, oral tacrolimus (Progaf®, Astellas Pharma Inc.) has replaced cyclosporine in many transplant centers (6) (Figure 1.2). Cyclosporine, like many other lipophilic drugs, suffers from low bioavailability and has been extensively studied for formulation methods to improve drug delivery. The Biopharmaceutics Classification System (BCS) Guidance classifies cyclosporine as a class IV drug, meaning that it is both poorly water soluble and poorly permeable. Despite a high affinity for hydrophobic molecules (log P = 2.92), cyclosporine remains poorly preamble due to its high molecular weight and rigid cyclical structure (11). Oral delivery of this drug is further hindered by P-glycoprotein-mediated efflux and extensive degradation by cytochrome P450 during first pass metabolism. Variability in bioavailability of orally administered cyclosporine in immunosuppressed patients has been shown to be more influenced by p-glycoprotien levels rather than intestinal enzymes (12). It has been suggested by some that limiting the role of p-glycoprotein action is an effective way to reduce variability in oral immunosuppressant absorption, consequently reducing incidence of acute graft rejection (13, 14). While metabolic rates may be effected by other medications (i.e. triazole antifungals, macrolide antibiotics, calcium channel blockers), the majority of research has focused on improvement of oral bioavailability through solubility enhancement, rather than chemically limiting the body's ability to metabolize these drugs.

The originally marketed oral cyclosporine product, Sandimmun® (Novartis), is available in soft gelatin capsules or as an oral solution dissolved in an ethanol/maize oil formulation. Bioavailability and resulting systemic concentrations of cyclosporine after

oral dosing of this oil based formulation was low due to its dependence on bile salt concentration in the upper small intestine (15). Bile salts are needed to elicit micellular solubilization of these oils, and because these salts may vary in concentration on inter and intra subject basis, the overall bioavailability of this product proved to be variable. An improved oral formulation was reported where in situ micoremulsification of cyclosporine occurs as soon as the formulation comes in contact with an external aqueous phase. By utilization of an emulsion stabilizing surfactant (DL-α-tocopherol) (16), Neoral® (Norvartis) is able to achieve therapeutic immunosuppressant levels with less variability. In a multicenter, double blind clinical study, efficacy in prevention of episodes of heart transplant rejection was shown to be superior in micoremulsified cyclosporine when compared to the older formulation. As would be expected, reduced variability in blood pharmacokinetic profiles using Neoral® was seen in the 1st year of treatment (17). Additionally, therapeutic blood targets were met with lower dosing of the microemuslion formulation, proving improved bioavailability. These results agreed with earlier findings reported by Tan et al in a single dose study in patients awaiting lung transplant suffering from cystic fibrosis. Overall bioavailability of microemulsified cyclosporine was shown to be 1.84 to 2.09 times higher (at 200 mg and 800 mg dose, respectively) than that of the conventional formulation in these patients (18).

Other novel oral cyclosporine drug delivery systems have been reported; many of which have not undergone substantial animal and human testing for efficacy and safety in lung transplantation. Engineered particles designed for delivery to lymphatic tissue have been reported by several groups in order to produce an enhanced effect on T lymphocytes. This strategy demonstrated moderate success in in vivo testing of engineered nanoparticles (19-21). Specifically, El-Shoabouri has shown increased absorption through the intestinal mucosa by incorporation of charge carrying polymers

which resulted in an increase in overall bioavailability of 73% when compared to microemulsified cyclosporine composition. Improvements in solubility and Caco-2 cell permeability have also been reported by incorporation of synthetic amphiphilic polymers, resulting in micellization of cyclosporine (22). Nanoparticle drug release was effectively targeted to the upper small intestine by incorporation of poly(methacrylic acid and methacrylate) copolymer and this showed an approximate 15% increase in bioavailability relative to Neoral®(23). Other attempts to control release and enhance cyclosporine solubility have incorporated the use of sodium lauryl sulfate-dextrin (24) and poly(lactic acid)-poly(ethylene glycol) (25) microspheres. Enhanced solubilites and rapid dissolution have been shown in poorly soluble drugs when amorphous particles are produced. Processes for making stabilized amorphous cyclosporine have been developed, including evaporative precipitation into aqueous solution (EPAS) (26, 27). Compositions containing polyoxyethylene (40) stearate dispersions (28) have also been reported to achieve comparable bioavailability to Neoral® using a solvent-melt technique to produce a stable amorphous powder. While generally unproven in complex biological models, these formulations should be considered when addressing the future of drug delivery systems developed for improving solid organ transplant rejection rates.

1.2.2 Tacrolimus

Orally administered tacrolimus is the cornerstone of immunosuppressive therapies for all forms of solid organ transplant. In the past 10 years, trends have shown oral tacrolimus use increasing as a maintenance therapy. In 2006, approximately 78% of lung transplant patients one year post-operative and approximately 73% of patients five years post-operative were using tacrolimus along with steroids and antimetabolites to prevent lung graft rejection (29). The replacement of cyclosporine as a calcineurin inhibition

agent with tacrolimus is based on data collected in both the laboratory and clinical setting. Specifically, patients receiving lung transplants over the course of a 3 year study were given immunosuppressive therapy with either orally administered cyclosporine or tacrolimus. In the first two years, survival rates were similar; however, early signs of rejection and cases of obliterative bronchiolitis were seen much more frequently in cyclosporine dosed patients (38% vs. 21.7%) (30). In a trial in the UK involving patients having received liver transplants, clinical outcomes were compared between patients dosed with tacrolimus (Prograf®) and microemulsified cyclosporine (Neoral®). Tacrolimus therapy was initiated at 0.1 mg/kg/day orally and adjusted appropriately to maintain blood trough levels of 5 -15 ng/mL, while cyclosporine was given orally at 10 mg/kg/day for maintenance of therapeutic trough levels between 150 ng/mL and 250 ng/mL. The reduced mortality rate and decreased need for retransplantation shown in this study after one year demonstrated the clinical advantage provided by tacrolimus as compared to cyclosporine. (31). Additionally, replacing oral cyclosporine with oral tacrolimus has been shown to improve pulmonary function in lung transplanted patients as determined by improvements in forced vital capacity and reduced exhaled nitric oxide levels (32). From a pharmacodynamic view point, these two drugs show almost exactly the same method of T cell inhibition; although improvements in therapeutic outcomes using tacrolimus may also be due to a potency that is 10-100 times greater than that of cyclosporine (30, 33). In addition to showing clinical improvements in daily maintenance therapy, oral tacrolimus has been shown effective as a rescue therapy in patients experiencing acute rejection while receiving oral cyclosporine.(34).

The innovator product, Prograf®, consist of a fine dispersion of amorphous tacrolimus in a hydroxypropylmethylcellulose (HPMC) carrier at 1:1 ratio and is intended for a twice daily dosing regimen. Superior drug solubility was reported for the Prograf®

formulation when compared to bulk, crystalline tacrolimus in dissolution testing due to improved tacrolimus solubility (35). Stabilized amorphous powders are often used in oral formulations as a strategy to enhance drug solubility (36). Overhoff et al successfully stabilized amorphous tacrolimus with a polymeric carrier by a rapid freezing technique and showed the ability to supersaturate dissolution media in in vitro testing. Further testing of oral dosing in a rat model showed greater systemic absorption when poloxamer 407 stabilized tacrolimus was dosed in comparison to Prograf® (37). In the granulated formulation of Prograf® intended for oral administration, lactose, croscarmellose sodium, and magnesium state are also included in capsules with the HPMC:tacrolimus combination. In the interest of enhancing patient compliance and reducing medication confusion for transplant recipients, a once daily tacrolimus formulation, Prograf® XL (formerly MR tacrolimus), has been developed. This once-daily (qd) formulation substantially reduced the maximum concentration (Cmax) of the tacrolimus in comparison to Prograf® by providing a more gradual drug release, while showing a similar extent of absorption (ratio of Prograf® XL to Prograf® for AUC0-inf = 97.3%) (38). Milligram-for-milligram conversion form twice daily tacrolimus to qd tacrolimus in clinical studies has been shown to produce equivalent systemic levels in kidney (39) (40) and liver (41) transplant patients in combination with other immunosuppressive therapies. Prograf® XL is associated with improved patient compliance due to reduced dosing frequency; however, variable gastrointestinal absorption with this formulation (also seen with the proprietary product, Prograf® and oral cyclosporin) necessitates close monitoring of trough blood levels.

Although much of the metabolic pathway is still not fully elucidated, tacrolimus is thought to undergo the same metabolic degradation as cyclosporine (12). When investigating tacrolimus absorption in the rat small intestine, it was found that the

presence of p-glycoprotein had an inhibitory effect on drug uptake, while the area of the intestine with a lower p-glycoprotein concentration, the jejunum, showed the highest absorption rates (42). Somewhat conflicting results were recently reported by Saitoh and coworkers. They found that tacrolimus is neither a substrate nor an inhibitor of p-glycoprotein and that cytochrome P450 was responsible for the drug's extensive metabolism (43). Unlike cyclosporine, tacrolimus is classified as a BCS class II drug, being poorly water soluble (4-12 µg/mL) (42), but highly permeable (log P = 3.3). Currently, some researchers are investigating other methods for enhancement of tacrolimus bioavailability, and many of the drug delivery technologies described for cyclosporine could also be applied for tacrolimus. Arima and coworkers have used various hydrophilic ß-cyclodextrins to enhance solubility in vitro as well as in a rat model, concluding that cyclodextrin solubilized tacrolimus showed a 1.2 fold increase in AUC compared to that of Prograf® (44). In a subsequent study, it has also been hypothesized that increased bioavailability of ß-cyclodextrin incorporated tacrolimus in a rat model is due to inhibition of p-glycoprotein efflux as well as increased solubilization. Drug loaded poly(lactide-co-glyolide) (PLGA) biodegradable nanoparticles and pH sensitive nanoparticles have also been investigated for colonic delivery in the treatment of inflammatory bowel disease (45, 46). These formulations also show potential for enhancement of systemic bioavailability in transplanted patients by targeting areas of the gastrointestinal tract where the drug is metabolized to a lesser extent.

1.2.3 Other agents

Many other oral immunosuppressive therapies such as corticosteroids and antimetabolites are used in the prevention of lung graft rejection (i.e. azathioprine, prednisolone) and are not confronted with the bioavailability obstacles seen in the

calcineurin inhibitors discussed above. These drugs, however, are limited in there clinical use and are often used in conjunction with other immunosuppressive agents because of their adverse side effects and narrow therapeutic windows (5). Some of these drugs do require special considerations when dosing, including mycophenolic acid and sirolimus. Mycophenolate mofetil, a prodrug commonly used as an antiproliferative agent, has been shown to cause irritation in the upper gastrointestinal tract when delivered orally due to the mofetil ester entity. Enteric coated mycophenolate sodium (Myfrotic®) is offered as an alternative that allows for drug targeting of the upper small intestine, avoiding gastric and upper intestinal irritation. Mycophenoate sodium has been shown to be effective in conjunction with other immunosuppressive regimens in prevention of solid organ graft rejection (47). Sirolimus, another immunosuppressive agent that blocks IL-2 proliferation, has been shown to be effective in transplant patients while avoiding nephrotoxicity normally seen with calcineurin inhibitors. Oral sirolimus (also known as rapamycin) has been shown to have a synergistic effect when dosed with cyclosporine or tacrolimus. Since both sirolimus and calcineurin inhibitors are metabolized by cytorchrome P450, increases in sirolimus bioavailability may be seen when co-administered due to reduced metabolism (12). This interaction should be noted by the clinician with doses adjusted accordingly. . Systemic toxicity (i.e. bone marrow suppression) and interstitial pneumonitis associated with oral dosing of sirolimus, has limited the widespread use of this agent in lung transplant patients.(48-50). Additional pulmonary complications associated with oral dosing of sirolimus such as lymphocytic alveolitis and alveolar hemorrhage have been reported to improve upon discontinuation or reduction of dose.

1.3 DELIVERY SYSTEMS INTENDED FOR THE PARENTERAL ROUTE OF ADMINISTRATION

Many immunosuppressive therapies are administered intravenously during patient hospitalization, especially in the case of induction therapy. Induction therapy is not advocated by all lung transplant centers, in fact it was preformed in less that 50% of lung transplant cases in recent years (29) (Figure 1.3). Induction therapy occurs post-operatively and involves dosing of powerful anti-lymphocyte antibody parenterals to achieve an initial level of immunosuppression. Critics of induction therapy site that "over suppression" might increase risk of infection, while advocates of this therapy believe it helps prevent acute rejection in the critical first few weeks (51). Intravenous immunosuppressants such as IL-2R antagonist (basilizimab, daclizumab) and polyclonal antibodies (anithymocyte globulin) are typically used in this therapy. As of yet, no clinical studies have reported data making a strong case for induction therapy in lung transplantation (52).

In cases of maintenance therapy, many marketed intravenous formulations are adaptations of the active used in oral compositions (as is the case for Prograf® and Neoral®). In Prograf®, polyoxyl 60 castor oil and dehydrated alcohol are used to solubilize tacrolimus. Sandimmune® for injection consists of cyclosporine in Cremophor® EL (castor oil) and alcohol solution. Due to toxicity and high risk of anaphylaxis associated with intravenous castor oil, these formulations are typically avoided in favor oral immunosuppression (53). Other formulations have been studied for prolonged drug release and reduced systemic toxicity through drug targeting. Injected liposomal cyclosporine (54) and tacrolimus (55) have been investigated in mice for their effect on therapeutic index and tissue targeting. Evidence of tissue targeting as well as prolonged systemic half lives was observed in both studies due to manipulation of fatty

acid chain length, surface charge, and particle size. Novel polymeric micelles of cyclosporine have also been studied and exhibited superior solubility, prolonged drug release, and lowered toxicity when compared with Cremophor® EL (Sandimmune®) solubilized injections (56, 57). Specifically, poly(ethylene oxide)-b-poly(ε-caprolactone) (PEO-b-PCL) micelles increased solubility and prolonged release of cyclosporine due to high encapsulation efficiencies and reduced molecular mobility within the micelle. Other methods of solubilizers such as ethanol and Cremophor® EL allow for higher molecular mobility and thus faster drug diffusion. In previous studies it was shown that cyclosporine for injection solubilized with Cremophor® EL causes hypersensitivity and toxicity in many patients. Additional studies have shown that Cremophor® EL also causes leaching from polyvinylchloride (PVC) tubing, delivering diethylhexylphthalate (a potential carcinogen) to the patient during intravenous administration. Due to these concerns with currently marketed intravenous cyclosporine, it is suggested that oral cyclosporine be considered as an alternative treatment (58, 59). Lactide and ε-caprolactone biodegradable microspheres loaded with cyclosporine were shown by Li and coworkers to potentially deliver steady levels of cyclosporine for several weeks while bypassing metabolic variations seen in convention oral dosing (60). After intramuscular injection in mice, similar results were seen as tacrolimus biodegradable microspheres maintained steady immunosuppressive blood levels for 2 weeks (61). By eliminating toxic solubility enhancers and slowing drug release rate, these types of extended release formulations become increasingly interesting for systemic immunosuppressive therapy.

1.4 DELIVERY SYSTEMS INTENDED FOR IMPLANTATION

Using similar prolonged release strategies, implantable polymeric devices have been designed for extended immunosuppressive release in corneal transplant patients.

Reservoir corneal implants of cyclosporine have been tested in equine and rabbit models, showing potential to provide sustained release for three to five years (62). Specifically, when glycolide-co-clatide-co-caprolactone was used as a polymeric carrier for sustained release of tacrolimus in a rabbit corneal transplant model, therapeutic drug levels were maintained for over 180 days (63). Similar results were seen for shorter release times with rapamycin loaded chitosan/polylactic acid nanoparticles in corneal transplanted rabbits (64). This methodology is interesting for solid organ transplant recipients as well; potentially allowing for long term localized therapy. Concern over invasive implantation procedures becomes a mute point in immunosuppression for transplant patients, since these implants could be inserted during transplantation surgery. Further concerns over implant safety and clearance/metabolic variability convey the need for additional studies in this method of immunosuppressive delivery.

1.5 Delivery Systems Intended for the Pulmonary Route of Administration

In an effort to localize immunosuppressive effects of calcineurin inhibitors, many groups have developed and investigated these drugs for inhalation. This strategy for targeting the deep lung tissue should provide high local drug concentrations, while limiting drug concentrations systemically; in turn reducing of toxic effects imparted by these medications. Cyclosporine, having been used for prevention of transplant allograft rejection for 25 years, was an obvious candidate for development into a formulation for aerosolization.

1.5.1 Cyclosporine

Early research in pulmonary targeted immunosuppression via cyclosporine aerosol delivery studied the drug's efficacy and tolerability by this delivery method. A basic formulation where pure drug was dissolved in ethanol was produced so that drug aerosolization could occur. While ethanol is used in many pMDI and nebulization formulations and is GRAS (generally recognized as safe) for inhalation at low levels, at high levels it has been shown to be irritating to the lungs and may cause changes in lung function (65). Regardless, cyclosporine in ethanol has been dosed in both animals and humans and shown promising results in comparison to traditional immunosuppressive therapy. In patients experiencing acute cellular rejection, aerosolized cyclosporine (300 mg) was administered as a rescue therapy daily for 10 days, followed by maintenance dosing at 3 doses per week. While standard rescue therapy of pulse methylprednisolone or equine lymphocyte immune globulin proved unsuccessful in all 18 patients, aerosolized cyclosporine was effective in 14 of the 18 (66). Additionally, patients displayed improvements in lung function and no significant change in renal function. In lung transplanted and non-transplanted experimental animal models, aerosolized cyclosporine showed superiority over intramuscular and subcutaneous injections (67, 68). At the same dosing level, the area under the concentration vs. time curve (AUC) of lungs in aerosol dosed rats was three times that of rats receiving intramuscular injection. In an allogeneic rat lung transplant model an 80% greater dose was required in injected rodents as compared to aerosolized rodents to prevent graft rejection. Furthermore, generally lower blood levels were seen in the aerosolized group at equivalent doses.

More recently, cyclosporine has been reformulated in propylene glycol, which is less irritating to the lung mucosa. In a 28 day safety study in rats and dogs, animals received as much as 2.7 times the equivalent maximum human dose (69). At these high

dosing levels, no unexpected systemic toxicity or respiratory toxicity was seen in either aerosolized cyclosporine in propylene glycol or propylene glycol alone. Additionally, cyclosporine lung concentration in rats was found to be 18 times than in the blood at given time intervals. Added viscosity of this solubilizing agent presents another caveat to dosing to the lungs in that a specific air-jet nebulizer and air compressor must be used. Increased viscosity in pulmonary formulations can lead to high variability in mass median aerodynamic diameter (MMAD), total output, and respirable fraction (RF%) between different nebulizer systems. Characterization of aerosolized cyclosporine in propylene glycol has been performed with the Aerotech II in combination with a high-flow compressor, showing a MMAD of 1.6 µm were 90% of the aerosol produced is below 5.0 µm (70). This method of aerosolization has been used in clinical trials of this formulation, ensuring consistent aerosol characteristics in the dose delivered to the patient. While clinical study samples size are small due to the infrequency of pulmonary transplant, several studies have been preformed for evaluation of this formulation in lung transplant recipients. In evaluation of the deposition and absorption rate of aerosolized cyclosporine, Burckart and colleagues concluded that the aerosolized drug displayed biphasic absorption shown by the rapid initial absorption into the blood ($t_{1/2}$ = 0.73hr) until 6 hrs after dosing when slower absorption after was noted ($t_{1/2}$ = 16.2hr) (71). It was hypothesized that the prolonged retention of cyclosporine in the lung might be due to macrophage uptake or lipophilic interactions between cyclosporine and phospholipids found in pulmonary surfactant and the alveolar membrane (72). Using radiolabelling and forced expiratory volume in one second (FEV1) , a linear relationship was developed showing a dose dependent relationship between post-transplant lung function and drug deposited in the lower airways. Improvement in lung function was seen over a 2 year span in patents where dosing resulted in 5 mg of drug deposition in the lung periphery

(73). In a study of patient survival after lung transplantation, conventional immunosuppressive therapy was given to 51 control patients, while 39 cases received cyclosporine aerosol in addition to conventional therapy. Estimates provided by Kaplan-Meier survival analysis showed a 4.5 year mean survival in patients receiving additional aerosol dosing, while the control group mean survival was only 2.3 years (74). In a large efficacy study involving lung transplant patients, a randomized single-center trial was performed involving 30 aerosol placebo dosed patients and 28 aerosol cyclosporine patients; all of which received oral tacrolimus, azathioprine, and prednisone for immunosuppressive management in addition to aerosol therapy. Results of this trial showed that, while having no significant effect on the incidence of acute allograft rejection, both patient survival (47% placebo vs 11% cyclosporine) and chronic rejection rates were significantly improved in the patients receiving aerosolized cyclosporine (75) (Figure 1.4). Interestingly, and contrary to previous studies, there seems to be no relationship in this study between acute rejection and chronic rejection in the aerosol dosed group. The authors theorize that since aerosolized cyclosporine contributes minimally to blood concentrations, there is less immunosuppressive effect on vascular lung tissue. Acute rejection, which is histologically characterized by lymphocytic infiltrates in and around vessels, might see little therapeutic effect with the addition of aerosol treatment.

The underlying issue in the FDA approval of what seems to be much improved targeted delivery method is the lack of and homogeneity and small size of patient populations in trials. With only 22 percent of lung transplant centers performing more than 20 transplants a year (29), the need for a unified multicenter effort becomes apparent. This, however; only compounds the problems of different immunosuppressive treatment protocols (i.e, induction, maintenance, adjuvant, and steroid therapies) and

imbalances between treatment and placebo groups (76). These issues, combined with a relatively small market outlook has led Novartis AG, the developer of this orphan drug, to sell rights to APT Pharmaceuticals (Burlingame, CA) who is expected begin Phase III trials this year.

In an effort to enhance the solubility of cyclosporine without the use of a lung irritating solvent or highly viscous agent, liposomal cyclosporine for inhalation was developed by Gilbert and coworkers (77). Preparation of cyclosporine in dilauroylphosphatidylcholine (DLPC) consisted of dissolving both excipients in t-butanol, followed by solvent removal and redispersion in water. In addition to solubility enhancement, liposomal delivery may offer the added benefit of drug retention in the lungs. Liposomal aerosol deposition in a dog model showed lung retention of approximately 120 min after a 25 mg dose (78).

1.5.2 Tacrolimus

As documented in laboratory and clinical studies, both oral and intravenous tacrolimus formulations have shown increased potency and efficacy over cyclosporine. Subsequently, many immunosuppressive protocols now have replaced the calcineurin inhibitor cyclosporine with tacrolimus. In keeping with this trend, researchers are now investigating the efficacy and safety of aerosolized tacrolimus in transplanted and non-transplanted animal models. The goal, as with inhaled cyclosporine, is to enhance immunosuppressive effects in the lung, while limiting toxicity systemically. In anticipation of the interest in using tacrolimus for targeted pulmonary delivery in transplantation as well as asthma therapy, Fujisawa (now Astellas Pharma Inc.) developed and patented a pMDI formulation containing tacrolimus, miglyol 812 (a lipophilic solubilizer), and HFA-227 (propellant)(79). In the first study incorporating

inhaled tacrolimus, Ingu and coworkers investigated the efficacy of aerosolized tacrolimus in lung allograft immunosuppression and compared with immunosuppression afforded by tacrolimus intramuscular injection (80). In both groups histopathological evaluation and suppression of cytokine expression denoted local immunosuppression in the transplanted lungs. As is the case with inhaled cyclosporine, inhaled tacrolimus was found to provide the same efficacy in the lung as intramuscular injection, while showing reduction in systemic blood levels (<0.5 ng/mL inhaled, 5.2 ng/mL intramuscular). Subsequent investigations have quantified tacrolimus deposition levels in the lungs by a micropartilce enzyme immunoassay method in transplanted rats with dose administered through intramuscular injection and inhalation. Significant immunosuppression was observed histologically in animals having a tacrolimus lung concentration of 270.4 ng/mL one hour after sacrifice (81). In addition to potential for lung transplant therapy, inhaled tacrolimus delivered by pMDI has also been investigated for inflammation reduction in egg-albumin challenged guinea pigs (82). Another formulation for aerosolization of tacrolimus has been studied by Schrepfer et al. Also in solubilized form, tacrolimus is aerosolized with 70% ethanol solution and studied for its inhibition of obliterative airway disease in tracheal transplanted rats (83-85). Much like previous formulations, findings demonstrated that aerosolized tacrolimus can achieve potentially immunosuppressive concentrations in the lungs, while avoiding excessive systemic levels. Upon histopathological examination, tracheal grafts did show immunosuppression in both oral and aerosol groups. Interestingly, upon cessation of aerosol and oral treatments, tracheal influx of inflammatory cells is much more rapid in the aerosol treated group due to lower systemic immunosuppression. Another novel formulation involving dispersed tacrolimus nanoparticles has been reported for prevention of lung allograft rejection via pulmonary delivery. This formulation offers the

benefit of supersaturating pulmonary fluid without the use of lung irritating solvents. In vitro efficacy was shown by lymphocyte suppression in mixed leukocyte culture (MLC) and mitogen stimulation assays (MSA) (86). A dispersion of tacrolimus and lactose (1:1) nanoparticles has been evaluated in vitro (as seen in Figure 1.5) and dosed to mice evaluated for blood and lung levels (87). Preliminary studies involving pulmonary dosing of this dispersion in a rat lung transplant model showed that high lung levels are achieved, while whole blood concentration remains low. Additionally, it was noted that drug concentration in transplanted allografts showed prolonged therapeutic levels in comparison to non-transplanted lungs.

Although no successful lung transplanted animal studies are shown in the literature, liposomal tacrolimus has been synthesized using dipalmitoylphosphatidylcholine (DPPC) for potentially targeting lung immunosuppression (88). As in pulmonary liposomal formulations of cyclosporine, tacrolimus liposomal formulations may allow for longer drug residence times after single dosing.

1.5.3 Corticosteroids

Oral or intravenous administration of corticosteroids in conjunction with a calcineurin inhibitor and antimetabolite has long been standard practice for maintenance immunosuppressive therapy. Inhaled corticosteroids used in the treatment of asthma and chronic obstructive pulmonary disease induce an anti-inflammatory response, suggesting possible application for immunosuppression in lung transplant patients. In spite of these observations, conflicting results have been seen in inhalation of corticosteroids for prevention of lung allograft rejection. Early success was seen in treating bronchiolitis obliterans syndrome (BOS) with inhaled budesonide and fluticasone (89, 90). More

recently, in a clinical trial investigating reduction of lymphocytic bronchiolitis in transplanted lungs, inhaled budesonide was shown to improve lung function (91); however, the absence of histological evaluation and long term patient success rates leave cause for further analysis. A more recent placebo-controlled clinical trial found that inhaled fluticasone propionate dosing twice daily for three months did not significantly prevent BOS in 30 lung transplant patients tested (92). Potential reasons for the inefficacy of this targeted therapy might have been the early onset of BOS (approx 6 months after transplant), known difficulties with pMDI use, or inadequate drug deposition in small airways. In a specific case cited by Naef and coworkers, co-medication of inhaled fluticasone propionate and prophylaxis itraconazole resulted in Cushing's syndrome in some lung transplanted patients. It was deduced that inhibition of cytochrome P450 by itraconazole reduced clearance of fluticasone, causing accumulation in lung tissue and increased blood concentrations (93). Based on these few studies, it becomes apparent that further work investigating early intervention with various corticosteroid formulations for prevention of BOS is necessary.

1.5.4 Antifungal prophylaxis

In addition to immunosuppression, lung transplant patients often require prophylactic antifungal therapy to assist a weakened immune system in defending against invasive fungal infections. Many of these treatments are delivered orally or intravenously for transplant recipients; however, novel inhaled antifungal formulations have been used to target infection in lung allografts. Abelcet®, a lipid complex of amphotericin-B, has been dosed to single and double lung transplant recipients to determine drug deposition. As expected, lower deposition was seen in the native lung of single transplant patients because of lower ventilation. Clinical data has shown that

Aspergillus infections may originate from the native lung in patients with single lung transplants. (94); consequently, therapeutic drug levels in this region are important. It is suggested that techniques for targeting lower ventilated areas should be evaluated to overcome this issue (95, 96). In another study, efficacy of pentamide prophylaxis was demonstrated in lung transplant patients for the prevention of Pneumocystis jeroveci (formerly carinii) pneumonia (97). This treatment was used instead of sulfamethoxazole-trimethoprim to prevent sulfa-related allergy. Future studies exploiting pulmonary delivery of antifungals in lung transplant cases are needed before this mode of therapy can be commonly used in clinical settings.

1.6 DELIVERY SYSTEMS INTENDED FOR THE SUBLINGUAL/BUCCAL ROUTE OF ADMINISTRATION

Cystic fibrosis, being one of the main indications for pediatric lung transplantation, is a disease that affects many organs, including the lungs and gastrointestinal tract. It is characterized by production of thick mucus in the lungs, recurrent pulmonary infections, and diminished pancreatic enzyme production. Consequently, orally administered lipophilic substances tend to be poorly absorbed through the intestinal mucosa. In the case of orally administered immunosuppressants after transplantation (i.e. tacrolimus, cyclosporine), a further reduced bioavailability might be expected (98). An alternative has been proposed to obtain therapeutic blood levels in transplanted patients by sublingual drug delivery (99). Due to variations in drug absorption after sublingual administration, only 67% of the blood samples taken in 6 lung transplanted patients with cystic fibrosis showed therapeutic levels. In a recent case report involving a kidney transplant patient, a contraindication arose that eliminated oral tacrolimus as a treatment option. For approximately one month, therapeutic levels

(between 7 and 15 ng/mL) of tacrolimus were targeted relatively successfully by taking the contents of oral tacrolimus and administering sublingually (100). The contents of oral tacrolimus (Prograf®), HPMC:tacrolimus matrix, could be expected to swell in the oral cavity and release amorphous tacrolimus into the saliva. Additionally, in pediatric liver transplants, buccal administration of tacrolimus suspension was used to promote patient compliance and prevent interference with gastrointestinal function. When compared to trough levels achieved by nasogastric tube (NGT) administration, buccal administration showed comparable therarpeutic levels in the target range (31% buccal, 24% NGT) (101). More investigation into this method of calcineurin inhibitor delivery is needed including design of a mucoadhesive controlled release formulation and comprehensive pharmacokinetic evaluation in animal models.

1.7 CONCLUSION

As new immunosuppressive chemicals are developed, delivery methods to maximize and target their therapeutic effect must be developed. In the case of lung transplantation, unique challenges confront clinicians as they try to avoid allograft rejection through suppression of the immune system, while not completely disabling the body's ability to protect itself from invasive infection. Likewise, unique opportunities for targeted drug delivery by pulmonary delivery may allow for local therapeutic effects, while limiting systemic toxicity. As further discoveries are made in new immunosuppressive chemical entities and subsequent investigations are conducted in safe and efficacious ways to delivery them, lung transplant patient quality of life and survival rates will continue to improve.

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Chapter 2: Characterization and Pharmacokinetic Analysis of Tacrolimus Dispersion for Nebulization in a Lung Transplanted Rodent Model

2.1 INTRODUCTION

Cases of end-stage lung disease such as severe chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and cystic fibrosis (CF) often show limited response to treatment and pharmacotherapy, leaving very few treatment options for clinicians. Lung transplantation has been accepted as an effective therapeutic option in the treatment of patients with end-stage pulmonary diseases. Annually, approximately 2,000 patients undergo lung transplantation when conventional treatments are unsuccessful. While surgical procedures and maintenance therapy can prove quite difficult, recent years have shown the frequency of lung transplant procedures to increase dramatically, nearly doubling in the last ten years (1). Much of this increase has been a result of improved patients outcomes resulting from more effective donor matching, advances in surgical techniques, and development of more effective rejection-preventing pharmaceuticals.

The introduction of a more potent immunosuppressive agent, tacrolimus, has been associated with increased success in the prevention of allograft rejection and bronchiolitis obliterans syndrome (BOS) compared to its predecessors (2, 3). Prograf, an oral tacrolimus formulation made by Astellas Pharmaceuticals, is used in maintenance therapy for approximately 70% of lung transplant patients one year after transplantation (4). While its use is off label (having FDA approval for only kidney, liver, and heart

transplant only), oral tacrolimus has been proven more effective as a maintenance (2) and rescue (5) therapy in lung transplant patients, while also showing improved patient pulmonary function in comparison to other treatments (6). Much of these improvements may be a consequence of tacrolimus being up to 100 times more potent than the alternative pharmaceutical, cyclosporine (7).

Advances in animal transplant procedures have improved scientific modeling of transplant-related complications in humans, and enabled testing of novel pharmaceutical products. Experimental lung transplantation programs are needed to improve key aspects of transplantation surgery, such as preservation, duration of ischemia, and ischemia–reperfusion injury. Many different experimental lung transplantation models have been used to date. Initially, the canine lung transplantation model was preferred (8), although since 1971, rats have become the model of choice (9, 10). Advantages of using the rat model include lower cost to the researcher as well as the availability of inbred strains which allow for discrimination between immunologic and non-immunologic events occurring after transplantation. In 1989, the use of cuffs in rat lung transplantation by Mizuta et al. showed improvements in the microsurgical technique compared to micro-suture anastamosis (11). In 1995, Reis et al. presented other significant improvements of the surgical technique (12), using Teflon cuffs instead of polyvinyl chloride or polyethylene cuffs which often leads to foreign-body reaction and fibrous tissue formation. Recently, Zhai et al. further improved the model by removing the holding tabs on the cuffs before insertion, decreasing the chance of kinking of the pulmonary artery, vein, and bronchus (13). The left orthotpic lung transplant on the rat is now performed by at least 10 research centers around the world.

In spite of these improvements in surgical technique and therapy, lung transplant rejection rates exceed those of all other forms of solid organ transplant, stemming form

the complexity of the pulmonary immune system and the frequency of exposure to foreign antigens. Further complexity is added to dosing regimens of immunosuppressive agents by the prevalence of nephrotoxicity, hypertension, and neurotoxicity (among others) at sustained and/or elevated systemic concentrations. Tacrolimus blood concentration has also demonstrated high intra-patient variation due to erratic gastrointestinal absorption and the low solubility. The delicate balance between therapeutic concentrations and toxic systemic concentrations combined with variable oral bioavailability requires frequent analysis of blood trough levels and often results in untoward complications. Clearly, a reduction in systemic drug levels accompanied by a pharmaceutical product with increased and consistent local bioavailability would alleviate many of the problems encountered in maintenance therapy.

The goal of targeting immunosuppressive therapy to the lungs and reduction of systemic side effects may be realized through the pulmonary delivery of tacrolimus. In transplanted animal models, others have demonstrated that aerosolized tacrolimus is effective in prolonging allograft survival, while maintaining systemic drug concentrations well below the toxic range (14-16). The objective of this study was to characterize a novel tacrolimus formulation for inhalation and determine its pharmacokinetics in a non-rejection lung transplanted rat model. Additionally, pharmacokinetic differences of non-transplanted and transplanted animals were studied. In comparing the drug levels of transplanted and non-transplanted animals, we hypothesized that no significant difference in tissue levels will be observed and that blood levels would remain below the therapeutic range.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Tacrolimus anhydrous (batch #070405) was purchased form Haorui Pharma-Chem, Inc. (Edison, NJ). Sirolimus (rapamycin) was bought from Spectrum Chemical (Gardena, CA). High Performance Liquid Chromatography (HPLC) grade acetonitrile, lactose monohydrate, zinc sulfate heptahydrate, barium hydroxide (0.3 N), and phosphoric acid (85%) were all purchased from Fisher Scientific (Fair Lawn, NJ). Heparin Sodium (10,000 IU/mL) was purchased from Baxter Healthcare (Deerfield, IL).

2.2.2 Formulation preparation

Ultra-rapid Freezing (URF) technology was used to produce tacrolimus powder for reconstitution in deionized water. This process is described in detail by Sinswat et al (17). Briefly, a 1 to 1 solution of tacrolimus to lactose monohydrate containing 0.75% w/v total solids was made by dissolving powders in a cosolvent mixture of acetonitrile and water. The transparent solution was applied to a rotating drum that had been cryogenically cooled to -50°C, where it was rapidly frozen (18). The frozen product was removed from the cryogenic surface and subjected to lyophilization using a VirTis Advantage Tray Lyophilizer (VirTis Company Inc., Gardiner, NY) to sublimate aqueous and organic solvents. After 40 hours of lyophilization, the product was packaged in a nitrogen rich environment at less than 20% relative humidity. Before aerosolization, tacrolimus powder for reconstitution was dispersed in deionized water by probe sonication.

2.2.3. Formulation characterization

2.2.3.1 Particle size analysis (Zeta, SEM)

Analysis of the particle size of tacrolimus colloidal dispersion was measured by laser scatter using a BI-ZetaPlus (Brookhaven Instruments, Holtsville, NY) and analyzed by a digital autocorrelator (Brookhaven Instruments, Holtsville, NY). Samples were run immediately after dispersion. Qualitative particle size analysis was conducted using a Hitachi S-4500 field emission scanning electron microscopy (SEM). Before imaging, dry samples were prepared by flash freezing tacrolimus colloidal dispersion in liquid nitrogen and adhered to a cooled SEM tray with carbon tape. The aqueous phase was then removed by lyophilization, leaving dried powder for analysis. Samples were gold-palladium sputter coated for 20 seconds under vacuum and read at 10 kV accelerating voltage.

2.2.3.2 Aerosol characterization

Pharmaceutical aerosols produced by an Aeroneb® Pro (Aerogen, Galway, Ireland) vibrating mesh nebulizer were characterized by cascade impaction and laser diffraction techniques. Aerosol impaction testing conducted with a Next Generation Pharmaceutical Impactor (NGI) (MSP Corporation, Shoreview, MN) was used to determine aerodynamic droplet size, dose emitted, and fine particle fraction. The NGI was operated at a flow rate of 30 L/min. Impacted formulation was collected with mobile phase and injected for HPLC analysis. The method used for HPLC analysis was adapted from Akashi and coworkers (19). A Waters 515 liquid chromatograph with a 996 PDA (Waters Corp. Milford, MA) combined with a Lichosphere RP C18 5 μm column (Varian Corp., Lake Forest, CA) was used for analysis. Mobile phase was composed of

acetonitrile, water, and phosphoric acid in proportions of 600, 400, and 1, respectively. Data collected was plotted in Sigma Plot (Systat Sofware Inc., San Jose, CA) and fit with a 3 parameter logistic curve for data interpellation. Mass median aerodynamic diameter (MMAD) was calculated based on drug collected on states 1 through 7 and micro-orifice collector (MOC) in accordance with USP 31 <601> guidelines. Fine particle fraction (FPF) was calculated as the percentage of the total emitted dose with an aerodynamic size less than 5 μm. A Malvern Spraytec (Malvern Instuments, Worcestershire, UK) was used to determine geometric droplet size distribution of colloidal dispersion and deionized water produced by the vibrating mesh nebulizer. An air current of 1 L/min was used to create the plume exiting the device.

2.2.4 Transplant study design

Adult male Lewis rats weighing 250 to 300 grams were used as both recipient and donor in the non-rejection model. All animals received proper care in compliance with an Institutional Animal Care and Use Committee (IACUC) approved protocol. Transplantation procedures were carried out using a cuff technique described by Mizuta et al (11). Prophylactic enrofloxacin (Baytril) was administered after transplantation to prevent possible infection. To confirm successful transplant and survival of allograft lung tissue, pathological review of the transplanted lung in preliminary animals was conducted. This evaluation verified whether or not a successful transplant had been accomplished in the non-rejection model, and that the dosing portion of the study could commence. Histology examples of tissues meeting evaluation criteria are given in Figure 2.1. During this study, a total of 24 rats received aerosolized tacrolimus, 12 having undergone unilateral left lung transplantation and 12 non-transplanted control rats. Each animal was given a nebulized dose of 6.4 mg of formulation dispersed in 3 mL

deionized water. The dispersion was nebulized by an Aeroneb® Pro and delivered via a four port nose-only dosing chamber (17). The animals were sacrificed 1, 6, 12, or 24 hours after the completion of single dose administration. Whole blood, obtained by cardiac puncture, was collected in heparinized conical vials, while the lungs, liver, spleen, and kidneys were excised and frozen in 20 mL scintillation vials.

2.2.4.1 Surgical Procedure

The donor rat was placed in the supine position and a tracheotomy was performed. A 14-20 gauge x 1 inch catheter (endotracheal tube) was placed via the tracheotomy site to facilitate continuous ventilation (Harvard rodent ventilator) using room air. Ventilation conditions consisted of a tidal volume of 2.5 to 3.0 ml and respiratory rate of 70 to 100 breaths/ min. A full sternotomy/celiotomy was performed via midline incision. Heparin (1000 units/kg) was then injected into the liver capsule. The inferior and superior vena cava was divided, the right and left atrial appendages were dissected, and a perfusion catheter was placed through a right ventriculotomy into the pulmonary artery. Cold Perfadex™ solution was perfused into the lungs as circulation ceased. The heart/lungs were harvested en-bloc after 20cc's of cold Perfadex had been perfused into the donor lungs under 10cm H20 pressure. The left lung was then procured by carefully dissecting out the pulmonary artery (PA), pulmonary vein (PV) and left main bronchus (Br). The PA, PV, and Br were cannulated with a Teflon cuff constructed from a 16G catheter for the PA and a 14G catheter for the PV and Br. Preparation of the cuffs began with cutting a 2.4 mm length and modifying to include a 1.5 mm extension (i.e., "handle") for manipulation. The cuff body was roughened with sandpaper to permit affixation. The PV was passed through the cuff, the proximal end was everted over the cuff and firmly fixed with a circumferential ligature of 6-0 silk suture. Preparation of the

Br and PA occurred in the same manner. The lung was then be wrapped in sterile gauze placed in a petri dish with Perfadex™ solution and placed on ice.

To prepare for recipient surgery, the orotracheal intubation was performed (14-20ga x 1.5" catheter) and the lungs were ventilated at room air under conditions of a tidal volume 2.5 to 3.0 ml and respiratory rate of 70 to 100 breaths/ min. Anesthesia for the recipient was maintained by inhalation of isoflurane to maintain comfort and immobility. The recipient then underwent a thoracotomy incision in the left third intercostal space and the native lung were weighted and pulled out of the chest, causing mild tension on the PA, Br, and PV and contributing to a smooth anastomotic procedure. The native PA, PV, and Br was then dissected and encircled with 6-0 silk suture using a microsurgical technique. A removable microclip was placed on each. Incisions were made with micro scissors into each of the three structures (PA, Br, and PV). The donor PA, followed by the donor Br and donor PV with attached cuff were inserted into the incisions made into the recipient PA, recipient Br, and recipient PV respectively. These anastomoses were then secured with the previously encircled 6-0 silk ligature suture in a circumferential fashion. The clips were released allowing full aeration and circulation of the lung donor graft. After pulmonary circulation was restored and the transplanted lung was functional, the recipient's native lung as well as the cuff extensions ("handles") were excised. The thorax was closed in layers. When the rat resumed spontaneous respiration, the orotracheal tube was removed.

2.2.4.2 Analysis of tacrolimus levels in whole blood

To determine systemic drug concentrations at each time point, a PRO-Trac™ II FK 506 Enzyme-linked Immunosorbent Assay (ELISA) kit (Diasorin Inc, Stillwater, MN) was used. In accordance with the supplied protocol, 50 mL of whole blood,

standards, and controls were each combined with 300 μL of digestion reagent in separate conical vials. After incubation, cellular components were centrifuged down and supernatant serum was removed. The samples were pipetted into a goat anti-mouse IgG coated 96-well plate along with anti-tacrolimus monoclonal antibody. After further incubation and addition of tacrolimus-horesradish peroxidase conjugate, the wells were rinsed with mild wash solution. Empty wells were filled with chromagen followed by acid stop solution to elucidate a colorimetric response. Spectrophotometric readings at 450 and 630 nm were recorded using a Bio-Tek® Instruments UV/Vis μQuant plate reader (Winooski, VT). A 4-parapmeter logistic fit was applied to the supplied standards and used to determine experimental concentrations.

2.2.4.3 Analysis of tacrolimus levels in lung tissue

Drug levels in lung tissue were quantified using liquid chromatography / mass spectrometry (LC/MS) after a liquid extraction procedure. Tissue was homogenized in 2 mL normal saline with 1.25 μg of internal standard (sirolimus). Extraction was facilitated by the addition of 0.3N barium hydroxide, 0.4N zinc sulfate heptahydrous, and acetonitrile. Centrifugation was used to separate tissue and precipitated proteins from dissolved tacrolimus. Supernatant was filtered, and lyophilized for 12 hours to remove all solvents. The remaining drug was reconstituted with mobile phase, filtered again through a 0.2μm PTFE filter, and prepared for spectrometric analysis. LC/MS equipment consisted of a Thermo Fisher Surveyor Plus HPLC system with PDA (Waltham, MA) and a Thermo Fisher LTQ FT Ultra Hybrid Mass Spectrometer (Waltham, MA). Other equipment specifications included, a C18 3μ Thermal Hypersil Gold 50 X 2.1 column and a gradient flow of acetonitrile and water (5:95 to 80:20 to 5:95).

2.2.4.4 Statistical analysis

Drug concentration in the lungs and blood of transplanted rats was compared to that of control rats at each time point. Analysis was conducted using Minitab® Release 14 statistical software (Minitab Inc., State College, PA). One way ANOVA followed by a post hoc Tukey test was used at p=0.05 to determine significant differences between transplanted and non-transplanted groups.

2.3 RESULTS AND DISCUSSION

2.3.1 Formulation

Tacrolimus, a lipophilic drug molecule (log P = 3.3) with poor water solubility (4 μg/mL), faces formulation hurdles in order to achieve high and reproducible bioavailability. Efforts to improve the oral bioavailability of tacrolimus have been made through the inclusion of dimethyl-β-cyclodextrin to in enhance solubility and reduce p-glycoprotein efflux (20) as well as microencapsulation to allow for sustained colonic delivery (21). To increase solubility, stabilization of the amorphous drug within a hydroxypropylmethylcellulose (HPMC) matrix was used and is market in the FDA approved oral formulation, Prograf (22, 23).

A similar strategy is applied in our novel pulmonary formulation in that the amorphous drug is stabilized; however, use of stabilizing excipients are limited to those that are generally recognized as safe (GRAS) for use in the lungs. In a formulation study involving URF particle engineering technology, it was found that a nanostructured solid dispersion of amorphous tacrolimus could be created using equal parts of lactose (17). In vitro testing of this drug product showed a 10 fold improvement in peak saturation of tacrolimus in simulated lung fluid. Pulmonary delivery the nanostructured powder

created by this process is facilitated by dispersion in an aqueous media, and nebulized by a vibrating mesh nebulizer. Particle size analysis of the resulting colloidal dispersion measured 5 minutes after addition to the aqueous phase showed a monodisperse distribution with a mean particle diameter of 239.2 μm. As seen in Figure 2.2, over the course of 30 minutes, the dispersion particle size and distribution changes, increasing in diameter and becoming more heterogeneous. The mean particle diameter grows from 240 to 352 μm in addition to a bimodal size distribution appearing at 30 minutes after dispersion. This phenomenon is not completely unexpected since the formulation is in a state of thermodynamic instability and contains no stabilizing polymers or surfactants to provide steric or ionic hindrance of particle interactions. Measured particle growth can be attributed to both particle aggregation and Ostwald ripening. Aggregation results as drug particle size is reduced and overall surface area of that mass is increased, leading to an increase in free energy of the system. Free energy is directly proportional to surface area as made evident by the following equation:

$$\Delta G = \gamma s/l \ * \Delta A \qquad (1)$$

where $\gamma s/l$ represents the interfacial tension of a substance and ΔA represents the change in surface area . In order to reduce the free energy and become a stable system, particles within a nanodispersion will aggregate to form larger particles with reduced surface area.

The second reason for measured particle size growth may be due to the actual crystal growth, not aggregation, of small particles, referred to as Ostwald ripening. In a thermodymaically supersaturated state, dissolved drug and excipient will begin to precipitate out of solution, nucleating to form small particles and adsorbing onto existing

ones. The rate of Ostwald ripening is dependant on mass transport due to molecular diffusion, interfacial surface tension, and the solute molar volume (24). This is the rationale for bimodal distribution that appears 30 minutes after powder dispersion.

Despite the aggregation and growth of particles over time, SEM images (Figure 2.3) and zeta potential measurements confirm that the primary particle size of the formulation immediately after dispersion is approximately 200 to 400 nm. To prevent particle size growth, nanodispersions for oral use will incorporate excipients such as povidones, pluronics, polysorbates, and cellulose derivatives; however, high levels of these stabilizers are generally not proven safe for pulmonary delivery, and can often result in alveolar epithelial damage (25). For these reasons, the colloidal dispersion used in this study was dispersed without stabilizing excipients. To limit changes in particle size distribution, all formulations were dosed within 15 minutes of preparation, before substantial particle growth and aggregation could occur.

In characterizing the nebulized aerosol produced by the Aeroneb® Pro, both cascade impaction and laser diffraction techniques were used. Since the nebulized drug product is a dispersion and not a solution, it was essential to study impaction results based on drug mass recovery to determine amount of drug actually delivered to the deep lung. The mass median aerodynamic diameter (MMAD) of the formulation measured according to NGI was 4.06 μm with a geometric standard deviation (GSD) of 2.7 μm. The aerodynamic size distribution of the aerosol is given in Figure 2.4. The FPF of the nebulized colloidal dispersion was 46.1%. Interestingly, volume median diameter (VMD), as measured by laser diffraction, for the same formulation was 6.55 μm. Theoretically, given that the dispersion media is water (unit density), all droplets produced should have identical aerodynamic and geometric diameter; however, testing differences may explain why the MMAD and VMD vary slightly.

NGI measurements taken according the USP 31 do not account for the droplet mass impacting in the induction port of the device, which can be assumed to represent droplets with aerodynamic diameters exceeding 11.7 μm (the cut off of the first stage). Laser diffraction analysis collects data on all droplets produced, including droplets larger than 11.7 μm in the calculation of VMD. When adjusted for the drug mass present in the induction port of NGI, the MMAD measured by impaction increases to 5.68 μm, much closer to want was measured by laser diffraction. Other impaction testing conditions may further influence the measured MMAD. Berg et al have studied the effects of temperature and humidity on partial droplet evaporation during impaction testing and concluded that under most ambient conditions (25°C, 50% RH) evaporation will occur (26). In order to prevent the skewing toward a lower MMAD, it is suggested that NGI testing be conducted at 5°C and 100% RH. Since characterization of tacrolimus colloidal dispersion occurred under ambient conditions, the possibility of underestimation of the MMAD due to evaporation is possible, and may account for the remaining difference between VMD and MMAD. Pan coating is another technique that is used to enable more accurate characterization by limiting aerosol "bounce" during NGI testing; however, this effect is thought to have a minimal contribution to liquid aerosol testing (27).

Laser diffraction measurements were used to compare the effect of the nanodispersion on aerosol generation and particle size distribution. As seen in Table 1, a slight increase in aerosol droplet diameter was seen after the inclusion of dispersed tacrolimus formulation. While surface tension has been shown to have minimal effect on the size distribution of aerosols produced by vibratory mesh nebulizers (28), an increase in droplet diameter may be due to the disruption of aerosol production caused by dispersed particulates in the aqueous phase.

2.3.2 Single dose transplant model

Therapeutic concentrations necessary to provide significant immunosuppression in the lungs have been shown in previous studies. Ide and coworkers determined that a tacrolimus concentration of 270 ng/g in the allografted lung is necessary to prevent transplant rejection (14). Similar concentrations were achieved in the allografted lungs in this study (399.8 ng/g) and resulting lung and blood levels over 24 hours were analyzed. As shown in Figure 2.5, peak total lung concentrations shown at 1 hr were 294.7 ± 10.5 ng/g and 301.5 ± 55.5 ng/g in transplanted and non-transplanted rats, respectively, showing no difference in overall lung deposition. Tracheal deposition was low, accounting for approximately 10% of total drug assayed 1 hour after dosing (Figure 2.6). It is uncertain whether mucociliary action would contribute to an increase or decrease of drug presence in the trachea since it would function to both relocate drug from the trachea into the laryngopharynx as well as add drug to lower regions of the trachea from the right and left bronchus. At the 6, 12, and 24 hour time points; however, mucociliary transit should have removed all solubilized and particulate drug in the mucus layer, meaning that only drug absorbed into tracheal tissue remains.

Assuming that lung levels 1 hour after dosing are an accurate representation of deposition level, average mass deposition in the right lung, 255.8 ng, was slightly larger than that in the left lung, 176.5 ng. Under normal breathing conditions a greater difference in deposition would be expected since the rodent right lung is on average twice as massive as the left, and is capable of higher tidal volumes. When tissue concentrations are compared, it is evident that deposition in the left lung is higher when adjusted for tissue weight (Figure 2.7). This discrepancy in lung concentration could be attributed to erratic breathing behavior. Disruptions in normal breathing in this study may have been due to animal excitement/anxiety during dosing, thoracic constrains of the dosing

chamber restraint tubes, residual pain from surgical procedures, or a combination of the three. This would result in a shallow-rapid breathing rate as described by Valberg et al (29). It has been noted that shallow breathing in rodents can lead to increased deposition in the central airways and heterogeneity in aerosol distribution (30). Since the right lung volume of the rat is much greater than the left, it is conceivable that shallow breathing may have a greater limitation on deposition in that right lung. The combination of shallow breathing with a MMAD that is slightly greater than the normal respirable range (1-5μm) may have magnified this deposition difference.

Blood concentration measured after dosing confirmed findings made my other authors that tacrolimus delivered directly to the lung produces minimal systemic drug levels. Specifically, in a lung transplant study where aerosolized tacrolimus was delivered via pressurized metered dose inhaler (pMDI), the ratio of tacrolimus concentration in the lungs to that in the blood was 55 to 1 one hour after the final dose (14). In a similar study conducted by Schrepher and coworkers, delivery of tacrolimus by pMDI to tracheally transplanted rats resulted a lung to blood ratio of 63 to 1 (15). When delivered orally in the same animal model, lung concentrations of 580 ng/g with a corresponding blood level of 67.1 ng/g were reached after 1 hour, giving a lung to blood ratio of only 9 to 1. As demonstrated in this rodent model, therapeutic lung concentrations are possible through oral delivery, however; it has been demonstrated in multiple clinical evaluations that excessive systemic concentrations will lead to untoward side effects, resulting in the reduction or elimination of the maintenance use of oral tacrolimus (31). Preferential accumulation of solubilized tacrolimus in the lungs may be due to its potential to associate with pulmonary tissue and phospholipids rather than partition into the aqueous media of the blood (32). Evidence of this preference has been observed with aerosolized cyclosporine, a drug molecule sharing a similar lipophilicity

(33). Specifically, cyclosporine dosed to human lung transplant patients showed biphasic absorption where initial absorption into the blood was followed by slow sustained partitioning into systemic circulation.

In this study, lung to blood ratios of 55 to 1 and 59 to 1 were shown in non-transplanted and transplanted rats, respectively. While results proved similar in all three studies, it has not been demonstrated in vivo if the drug deposited in the lungs is indeed bioavailable, or whether it is merely present in particulate form on lung epithelia. Characterization of tacrolimus colloidal dispersion in vitro provides substantial evidence that the drug is in fact bioavailable on the epithelial surface. Enhanced solubility in simulated lung fluid stemming from the nano-amorphous nature of this drug product has been demonstrated in previous studies and may impart superior drug availability to the lung epithelial layer in comparison to crystalline and/or micronized drug products. Future animal studies are needed to confirm that the enhanced solubility in simulated lung fluid translates to improved solubility and drug absorption in vivo.

It is well documented that transplanted lung allografts exhibit multiple postoperative complications after reperfusion (34). While most clinical complications arise from injury due to inflammatory cascades, physiological problems also exist in the basic functionally of the lung tissue. Disruptions of ciliary action, lymphatic clearance, and surfactant composition have been noted in multiple cases of animal and human lung transplant. In addition to affecting lung function and immunogenicity, these areas of dysfunction may cause changes in the pharmacokinetic profile due to impairment of drug solubility and particulate clearance. For the transplanted rat model investigated in this study, it was evident that tacrolimus is retained in the transplanted lung due to physiological disruptions caused by transplantation surgery. Six hours after the dose was administered, a statically significant difference ($p<0.05$) was observed between the left

lung concentration of control and transplanted animals (Figure 2.7a). While the exact mechanisms of drug retention in the transplanted lung are unclear, it is reasonable to assume that one or a combination of the physiological disruptions mentioned may explain the pharmacokinetic disparity.

In the upper airway, disruption of the mucociliary escalator due to denervation of the lung effects clearance of particulate matter, making the lungs more susceptible to nosocomial infection (35, 36). This may also contribute to prolonged retention of solid and semi-solid aerosols in bronchoalveolar tissue of transplanted lungs. Macrophage uptake and subsequent translocation of drug particulates to the lymphatics of the lung may also contribute to drug retention in transplanted tissue given that lymphatic circulation is also impaired (37). Reduced functionality of the alveolar macrophage itself has also been recognized after lung transplantation (38). Finally, heightened insolubility of the drug formulation in pulmonary fluids may also contribute to retention of drug within transplanted lungs. Previously, it has been shown that amorphous tacrolimus can achieve concentrations in excess of 70 μg/mL in simulated lung fluid (17); however, the epithelial fluid of the transplanted lung has been shown to contain decreased fractions of pulmonary surfactant (i.e. dipalmitoyl-phosphatidylcholine (DPPC)) (39). Wetting and eventual dissolution of drug particles could be reduced in an environment where this surfactant is not present and the surface tension of the fluid layer is increased (15.8 mN/m v 3.4 nN/m). If tacrolimus drug particles deposited in the transplanted lungs show a reduced solubility when compared to those deposited in normal lungs, retention of the drug in the airway would be expected, particularly if cellular clearance is correspondingly impaired.

2.4 CONCLUSION

It is clear that tacrolimus colloidal dispersion can be effectively nebulized to the lungs of lung transplanted rats to obtain therapeutic local concentrations. As hypothesized, resulting systemic concentrations remained low, not exceeding 6 ng/mL after a single dose and avoiding potentially toxic levels. Although it has not been demonstrated in vivo whether or not tacrolimus colloidal dispersion remains as particulates in the airway or is fully bioavailable as a solution in pulmonary fluid, previous studies have demonstrated in vitro that the formulation possesses the capability of supersaturating simulated lung fluid (17). This suggests that tacrolimus colloidal dispersion may be more locally bioavailable than previously studied formulations. This combined with the natural lipophilicity of the molecule may allow for high pulmonary bioavailability with low resulting system drug levels, as shown in this study.

Surprisingly, absorption of drug from the transplanted lung showed a slight, but significant retention of deposited drug. Denervaition and physiological changes in transplanted tissue are most likely the reason for this pharmacokinetic difference.

Several studies have shown the benefits of localized therapy in pulmonary delivery for maintenance or rescue immunosuppression in lung transplant recipients. Tacrolimus, a more potent and clinically effective immunosuppressive agent, is the therapy of choice for lung transplantation; although it presents some formulation challenges. Using solubility enhancing technology adapted with a method of localized therapy may prove to further enhance the therapeutic potential of tacrolimus for lung transplant recipients.

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Chapter 3: Preclinical Safety Evaluation of Tacrolimus Dispersion for Nebulization

3.1 INTRODUCTION

Immunosuppressive therapy has become increasingly important in the success of solid organ and bone marrow transplantation over the past 25 years. With advances in donor matching, continued surgical innovation, and the introduction of calcineurin inhibiting immunosuppressive drugs, the likelihood of allograft rejection has been substantially reduced, increasing a patient's probability of survival and quality of life. Currently, lung transplantation is the least successful of all common solid organ transplant procedures. Despite the improvements in surgical techniques and development of more potent immunosuppressive drugs, the survival rate of lung transplant patients 5 years post-op is only 50 percent (1). Many of the complications associated with treatment and maintenance dosing of lung transplant recipients can be attributed to the delicate balance that exists between proper immunosuppression of the lungs and prevention of untoward side effects and opportunistic infection. The immunological function of the lung is poorly understood and unique from any other organ of the body (2), complicating diagnosis and treatment of many immune-related lung diseases. Generally, immunosuppression of the allografted lung is achieved through oral dosing of a three drug regimen consisting of a calcineurin inhibitor, purine synthesis inhibitor, and corticosteroid(3). Maintenance dosing is continued for the rest of the patient's life and does not routinely involve corticosteroids due to side effects associated with prolonged

administration. Tacrolimus (TAC) and mycophenolate mofetil are the most common calcineurin inhibitor and purine synthesis inhibitor used for maintenance therapy.

A more logical approach for pulmonary immunosuppression is to administer the therapeutic agent directly to the lung via an inhalable aerosol. Researchers have investigated the delivery of an aerosolized calcineurin inhibitor, cyclosporine A (CSA), in animal models for determination of its efficacy and safety in lung tissue. In a safety study in rodents and canines, Wang and coworkers determined that inhaled cyclosporine presented no detrimental effects on pathology or lung function, even at 2.7 times the proposed human dose (4). This strategy has been investigated by various clinicians as well in hopes to improve maintenance therapy and prevent lung transplant rejection (5-8). Clinical trials involving lung transplant recipients have shown that while no statistical improvements in acute graft rejection were seen when compared to inhaled placebo, prevention of chronic rejection, and ultimately patient survival, were significantly improved with aerosolized CSA (7). In these trials, nebulization of CSA was facilitated by dissolving it in a viscous, non-toxic solvent, propylene glycol. While recognized as safe for use in pulmonary drug products, high levels of propylene glycol have been shown to cause mild to moderate irritation and cough in a majority of patients, and may require the use of a local analgesic before administration (7). Additionally, the high viscosity of propylene glycol may prevent proper aerosol production with certain nebulizers, changing aerosol characteristics and possibly limiting deep lung deposition (9). These factors, as well as a relatively less potent therapeutic effect, may reduce the inclination for clinical acceptance of this therapy.

Tacrolimus, first commercialized by Fujisawa, Inc in 1990 as Prograf®, elicits a therapeutic response by inhibiting the functionality of calcineurin and has been shown to be 10 to 100 times more potent than CSA (10). Clinical comparisons of oral CSA and

TAC in solid organ transplant patients have concluded that TAC improves both short term graft success (11, 12) and overall patient survival (13). In a pharmacoeconomic comparison, TAC was shown to reduce the associated healthcare and therapy costs by at least 25% in kidney transplant patients when compared to CSA (14). Other indications where TAC has been investigated for reduction of inflammation include atopic dermatitis (Protopic®), ulcerative colitis, graft-versus-host disease, and asthma (15).

While TAC therapy has noted success clinically and is currently more widely administered for transplantation than CSA (16), a variety of adverse side effects are associated with elevated systemic concentrations over prolonged periods of therapy. Among the most severe and commonly occurring side effects associated with oral TAC are nephrotoxicity, neurotoxicity, hypertension, diabetes mellitus, and increased risk of opportunistic infection; all of which have shown some evidence of dose dependence. Typically, neurotoxicity due to TAC maintenance therapy will result in mild effects such as headache or tremor. Cases of TAC-induced severe neurotoxicity are rare; however, they can lead to symptoms such as seizure, delirium, or coma and are commonly enabled by a reduction in p-glycoprotein efflux function (17). High sustained systemic concentrations of TAC can also lead to new onset diabetes mellitus (NODM). In a recent study analyzing reports form the past decade of calcineurin inhibitor-induced NODM, evidence showed that TAC was found to be more likely to cause this disease than CSA (18). A higher concentration of FK506 binding protein (FKBP-12) in the pancreas may explain the greater incidence of NODM in patients receiving TAC. In comparison to therapeutic maintenance therapy with CSA, cardiovascular events associated with TAC are significantly reduced (19). Close monitoring of trough blood concentrations is critical among patients using oral TAC to avoid severe side effects normally associated with blood levels above 50 ng/ml and to monitor variability of intestinal absorption.

In order to avoid systemic side effects that might lead to dosing protocol complications, additional medication, and elevated patient discomfort, a method of drug delivery in lung transplant patients providing a reduced systemic TAC concentration is needed. Additionally, local concentrations exceeding those provided by current oral therapy could potentially increase pulmonary therapy, resulting in enhanced suppression of the local immune response. This study investigates the safety and pharmacokinetics of once-daily TAC colloidal dispersion in a rodent model over 28 consecutive days. Blood chemistry, complete blood cell count, and tissue histology were conducted to evaluate the physiological effect of the dosing regimen. Evaluation of the stability of TAC powder for reconstitution is also reported. By providing organ-targeted therapy, multiple doses of TAC should provide heightened lung concentration, while reducing toxicity-inducing systemic blood levels.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Tacrolimus anhydrous (batch # 070405) was purchased from Haroui Pharma-Chem (Edison, NJ). High Performance Liquid Chromatography (HPLC) grade acetonitrile, lactose monohydrate, zinc sulfate heptahydrate, barium hydroxide (0.3 N), magnesium chloride, sodium phosphate dibasic anhydrous, sodium sulfate anhydrous, calcium chloride dihydrate, sodium acetate trihydrate, and phosphoric acid (85%) were all purchased from Fisher Scientific (Fair Lawn, NJ). Sodium chloride and normal buffered formalin solution (10%) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Potassium Chloride was purchased from EM Industires, Inc. (Gibbstown, NJ). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar-Lipids, Inc.

(Alabaster, AL). Sterile 0.9% Sodium Chloride for injection, USP was purchased from Hospira, Inc. (Lake Forrest, IL). Heparin Sodium (10,000 IU/mL) was purchased from Baxter Healthcare (Deerfield, IL).

3.2.2 Formulation composition and preparation

TAC powder for reconstitution was prepared using the Ultra-rapid Freezing (URF) process described by Sinswat (20). Equal amounts of TAC and lactose are dissolved in acetonitrile/water (60/40) so that the total solids content of the formulation is 0.75% w/v. The solution is applied to a stainless steel rotating drum that has been cryogenically cooled to -50°C. The frozen solution is collected in liquid cryogen and freeze dried using a VirTis Advantage Tray Lyophilizer (VirTis Company Inc., Gardiner, NY) to remove water and organic solvent. A detailed explanation of this particle engineering technology is given by Overhoff et al (21). Product was packaged under nitrogen at 25°C and less than 20% relative humidity.

3.2.3 In vitro characterization

3.2.3.1 Powder x-ray diffraction (PXRD)

X-ray diffraction patterns were determined for the drug product stored for up to 3 months using a Philips 1710 X-ray diffractometer with a copper target and nickel filter. Leveled powder was measured from 15 to 55 2θ degrees using a step size of 0.05 2θ degrees and a dwell time of 2 seconds.

3.2.3.2 Dissolution testing

Evaluation of the ability of formulated powder to supersaturate simulated lung fluid (SLF) was performed using a modified United States Pharmacopiea (USP) 31 Type 2 dissolution apparatus. Five small volume vessels were filled with 97 mL of SLF with 0.02% DPPC, maintained at 37°C, and stirred at 100 rpm. Preparation of SLF was carried out as outlined by Davies et al (22). Addition of 0.02% DPPC was performed by creating a thin lipid film via rotary evaporation with a Buchi Rotavapor R-210 (Flawil, Switzerland) and dispersing in SLF at 50°C (23). Five aliquots of 20 mg TAC powder for reconstitution were weighed, placed in 20 mL scintillation vials, and dispersed in 3 mL de-ionized water. Dispersion was facilitated by probe sonication with a Branson Sonifier 450 (Danbury, CT) (output 6, 10% interval) for 2 minutes under cooled conditions. Immediately after dispersion, each vial was added to a respective dissolution vessel. Aliquots of 2 mL were drawn at 5, 15, 30, 60, 120, and 240 minutes and filtered through 0.2 μm PTFE teflon syringe filters (National Scientific, Rockwood, TN). Subsequently, 1 mL of the filtrate is added to 1 mL HPLC mobile phase, consisting of acetonitrile, water, and phosphoric acid in a ratio of 600:400:1. Quantitation of each sample is performed by HPLC according to a method adapted from Akashi et al (24). A Waters 515 liquid chromatograph with a Water 996 Photo Array (Water Corp. Milford, MA) outfitted with a Lichosphere RP C18, 4 mm X 250 mm, 5 μm column (Varian Corp., Lake Forest, CA) was used for analysis. Analysis conditions such as flow rate, column temperature, and injection volume were 1.5 mL/min, 50°C, and 20 μL, respectively. Tautomers of TAC eluted at 8.3 and 13.2 minutes, while the main drug peak eluted at 17 minutes. All three peaks were measured at 215 nm and represent the active moiety, therefore all three were used in quantitation. Standards, prepared in mobile phase by serial dilution, ranged in concentration from 100 to 3.125 μg TAC per

mL. Standard curve linearity ($r^2 = 0.999$), system precision (RSD = 1.9%), and accuracy were validated for this method.

3.2.3.3 HPLC Testing

Five concentrations, 5, 2.5, 1.25, 0.625, and 0.3125 mg/mL, of bulk TAC in HPLC mobile phase were evaluated for purity using the HPLC method explained in section 3.3.3.2. Noise contribution from the solvent front was determined by blank injection and was seen to affect the chromatograph baseline until approximately 3.5 minutes after injection. All detectable non-tacrolimus peaks occurring after the solvent front were considered impurities and compared to tacrolimus peaks for determination of percent impurity. The effect of processing, excipients, and storage on TAC purity in the drug product was also evaluated. Product purity was evaluated immediately after production and after 1, 2, and 3 months storage in sealed packaging at room temperature. Samples containing between 4 and 5 mg of drug product were dissolved in 2 mL mobile phase and analyzed by HPLC. Two samples were reconstituted and analyzed for each month storage. For identification of potential degradants, a battery of forced degradation tests were performed on the bulk drug in accordance with the guidelines presented by the International Conference on Harmonization Q3B(R2). Acidic, alkaline, oxidative, and thermal degradation were each induced to a 5 mL solution containing 1 mg TAC in acetonitrile. Acid degradation was accomplished by adding 10 mL 1N HCl and incubating for 1 hr at room temperature. The reaction was quenched by the addition of 10 mL 1N NaOH. Similarly, base degradation incubated the samples for 1hr with 10 mL 1N NaOH. The reaction was quenched with 10 mL 1N HCl. Oxidative degradation involved the addition of 1 mL 30% H2O2 to the solution and incubating for 1 hr. Thermal degradation was completed by incubation in a 60°C water bath for 6 hours. At

the conclusion of quenching and/or incubation, all samples were diluted to 100 mL with water. Two milliliters of the diluted sample was filtered through a 0.2 μm PTFE teflon syringe filter. One milliliter was then added to 1 mL mobile phase and injected for HPLC analysis. Degradation peaks were identified and quantitated by the HPLC method outlined in section 3.2.3.2.

HPLC was used to analyze the drug product potency after 1, 2, and 3 months storage. Samples of between 4 and 5 mg of drug product were dissolved in 2 mL mobile phase and analyzed by HPLC. Two samples were reconstituted and analyzed for each month storage. Based on manufacture and batch formula detailed in section 3.3.2, theoretical potency is presumed to be 50% of the total drug product weight.

3.2.3.5 Residual solvent analysis

Gas chromatography (GC) performed with a Hewlett Packard 5890 Series II GC (Hewlett Packard, Palo Alto, CA) was used to determine the drug product residual solvent levels according to a revised USP 467 method for class 2 solvents. A Zebron™ ZB-624, 30m, 0.32 id, 1.8 μm column (USP phase G43) (Phenomenex, Torrance, CA) was used at a helium flow rate of 2.2 mL/min. Injection temperature as set at 140°C and oven temp was set to 40°C for the first 10 minutes then increased to 240°C for another 10 minutes. Flame ionization detector (FID) temperature was set at 240°C. Standards for injection contained a concentration of 4, 2, 1, 0.5, and 0.25 μL/mL acetonitrile (ACN) in dimethyl sulfoxide (DMSO). ACN peak elution occurred 6.15 minutes after injection.

3.2.4 In vivo tolerability study in a rat model

3.2.4.1 Pulmonary dosing method and regimen

TAC colloidal dispersion was administered to healthy male and female Sprague Dawley (SD) (Harlan Inc., Indianapolis, IN) rats in clean, humane conditions and in accordance with an Institutional Animal Care and Use Committee (IACUC) approved protocol. Aerosol was produced by vibrating mesh nebulization using an Aeroneb® Pro (Aerogen, Galway, Ireland) and delivered to the animals via a four port nose-only dosing chamber (20).

Eight female and 8 male rats were given treatments containing TAC (active group) for 28 days, while 4 male and 4 female rats were given equivalent volumes of normal saline (control group) for the same period. Six rats in the active group and 4 of the control group were maintained for one week after the last dose in order to evaluate resolution of any pulmonary inflammation that might occur after 28 days of active treatment. All other animals were sacrificed 24 hours after the last dose was administered. Eight animals in the active group were designated for determination of pharmacokinetic parameters. All other animals were designated for histopathological evaluation.

Before nebulization, 6.4 mg TAC powder for reconstitution was dispersed in 3 mL deionized water as described in section 3.3.3.2. Rats were exposed to the aerosolized dispersion for approximately 10 minutes, or until completion. Once daily aerosol administration was performed at the same time each day for 28 days. Animals were sacrificed by isoflurane (over 10%) inhalation and subsequently confirmed with cardiac puncture. Approximately 3 mL of whole blood was drawn for pharmacokinetic analysis and hematological testing. Blood for pharmacokinetic analysis was placed in 1.5 mL conical tubes containing 20 μL of 10,000 IU/mL heparin sodium. Lung tissue was

excised from designated rats and frozen at -20°C until determination of TAC levels by tissue extraction. Liver, spleen, kidney and lung tissue was excised and fixed in 10% formalin solution for histological investigation.

3.2.4.2 Analysis of tacrolimus levels in whole blood

Heparinized whole blood samples were evaluated for TAC levels using Enzyme-linked Immunosorbent Assay (ELISA). A PRO-Trac™ II FK 506 ELISA kit was purchased from Diasorin Inc (Stillwater, MN) and used according to the manufacturer's protocol. Fifty microliters were taken from each of 5 standards (ranging from 0.3 ng/mL to 30 ng/mL), 2 controls, and all whole blood samples and added to a 1.5 mL conical tubes along with 300 μL reconstituted digestion reagent. Each tube was mixed, then incubated for 15 minutes at 75°C. To separate digested cellular contents, centrifugation was conducted at 1800 g for 10 minutes, after which 100 μL of supernatant were added to duplicate wells of a goat anti-mouse IgG coated 96-well plate. Fifty microliters of anti-tacrolimus monoclonal antibody were added, followed by plate incubation and shaking at room temperature and 700 rpm form 30 minutes. After incubation, 50 μL of tacrolimus-horseradish peroxidase conjugate were added and the plate was shaken again for 60 minutes at 700 rpm. Liquid contents of the wells were then removed by washing, and 200 μL chromogen were added, and shaken again for 15 minutes at 700 rpm. The reaction was stopped by 100 μL of acidic stop solution and the plate was immediately read at dual wavelengths of 450 and 630 nm on a Bio-Tek® Instruments UV/Vis μQuant plate reader (Winooski, VT). The standard curve was plotted using Sigma Plot Systat Software Inc. (San Jose, CA) and fit with a 4-parameter logistic curve.

3.2.4.3 Analysis of tacrolimus levels in lung tissue

TAC was extracted from rodent lung tissue by a liquid extraction procedure and quantitated using liquid chromatography / mass spectrometry (LC/MS). Before tissue homogenization, 1.25 μg of internal standard (sirolimus) dissolved in 1 mL acetonitrile was added to the tissue. Two milliliters of normal saline were added to the lung tissue before homogenization. Lung tissue was homogenized in a scintillation vial for approximately 5 minutes at cooled temperatures. To extract TAC from bound tissue proteins, 0.1 mL of 0.3N barium hydroxide, 0.1 mL of 0.4N zinc sulfate hepahydrous, and 7 mL acetonitrile were added to the homogenate. The homogenate was vortexed for 30 seconds and placed in 15 mL polypropylene centrifugation tubes (Fisher Scientific, Fair Lawn, NJ). Centrifugation was conducted for 15 minutes at 3000g. Supernatant was then removed and filtered through a Whatman 0.45 μm nylon syringe filter (Fisher Scientific, Fair Lawn, NJ). Water and acetonitrile were removed from the supernatant by 12 hr lyophilization ramping from -40°C to 25°C after freezing the sample. The resulting solids were reconstituted in 2 mL acetonitrile and centrifuged again at 3000 g for 15 minutes. One milliliter of the supernatant was taken for injection and analysis by LC/MS. A Thermo Fisher Surveyor Plus HPLC system with PDA (Waltham, MA) was used in combination with a Thermo Fisher LTQ FT Ultra Hybrid Mass Spectrometer (Waltham, MA) for sample analysis. The system was outfitted with a C18 3μ Thermal Hypersil Gold 50 X 2.1 column, injected 10 μL of each sample, and run at a flow rate of 10 μL/min. Gradient flow was of acetonitrile and water were used where acetonitrile and water concentrations were 5% and 95% initially, changed to 80% and 20% at 17 min, and gradually ramped to back to 5% and 95% at 20 minutes, respectively. TAC was quantitated by integration of peaks occurring between 825.5 and 827.5 m/z.

3.2.4.4 Complete blood count and serum chemistry

After cardiac puncture, approximately 1 mL of blood was used for complete blood count (CBC) and serum chemistry. Samples were drawn and the needle was removed to prevent excessive red blood cell lysing. Lavender-topped BD Microtainer® tubes with K_2EDTA (VWR Scientific, West Chester, PA) were filled between 250 µL and 500 µL. Each tube was then inverted 10 times to ensure thorough anti-coagulant mixing. For serum chemistry analysis, whole blood samples between 400 µL and 600 µL were added to yellow-topped BD Microtainer® SST tubes (VWR Scientific, West Chester, PA), inverted 5 times, and allowed to remain at room temperature to coagulate for 30 min. Coagulated blood is then centrifuged at 6000 g for 90 seconds. After centrifugation, blood serum was pipetted into a 1.5 mL conical vial. Both CBC and serum chemistry samples were kept at 4°C until analysis. Analysis was run within 24 hours of blood draw by IDEXX preclinical research services (Westbrook, ME).

3.2.4.5 Tissue histology

At completion of the dosing regimen, lung, kidney, liver, and spleen tissue of both control and experimental groups were reviewed by two different blinded examiners. Eight rats having received 28 days of once daily TAC colloidal dispersion were evaluated; four were sacrificed 24 hours after the final dose, the other four sacrificed 168 hours after the final dose. Eight control rats followed the same dosing regimen and received aerosolized normal saline instead of TAC. Lung tissue was instilled with 5 mL of 10% formalin through cannulation of the trachea, submerged in 40 mL 10% formalin, and embedded in paraffin wax. Lung sections were stained with haematoxylin and eosin (H&E), cluster of differentiation 68 (CD 68), and periodic acid Schiff (PAS) and evaluated. Kidney, liver, and spleen were also submerged in 40 mL formalin and fixed in

paraffin wax. Non-lung tissue sections were stained only with H&E. Histopathologic inflammation scores were assigned according to the criteria outlined by Cimolai et al (25).

3.2.4.6 Statistical analysis

Statistical analysis of CBC and serum chemistry results was run using Minitab® Release 14 statistical software (Minitab Inc., State College, PA). One way ANOVA followed by a post hoc Tukey test was used at p=0.05 and p=0.01 to determine significant differences between control and experimental groups.

3.3 RESULTS AND DISCUSSION

3.3.1 In vitro characterization

An amorphous nanoparticulate drug formulation offers advantages over crystalline drug products in that bioavailability may be enhanced by increased solubility, reduced drug particle size, and improved wetting. The marketed oral formulation of TAC, Prograf®, uses this rationale by providing improved solubility through a solid dispersion of TAC and hydroxypropyl methylcellulose (HPMC) (26). For purposes of pulmonary delivery, lactose is a more attractive choice for stabilization of TAC due to its recognition by the FDA as a safe excipient for pulmonary products. Additionally, lactose is water soluble and has been used as a lyoprotectant (27), making it likely to provide stability to amorphous TAC upon storage. When added to aqueous media, the solubilization of lactose and thermodynamic instability inherent in amorphous TAC results in a supersaturated solution during the initial stages of dissolution. After approximately 30 minutes, supersaturated TAC comes out of solution to form the more

kinetically favored precipitated state. In order to determine the stability of our amorphous drug product for reconstitution over 3 months at the conditions previously described, PXRD and supersaturation dissolution testing were conducted. It was determined that the drug formulation maintained its amorphous nature throughout the 3 month period. Figures 3.1 and 3.2 show that amorphous TAC is still present throughout the 3 month period and that the capacity to supersaturate SLF is still present. It is clear that a ratio of 1:1, lactose to TAC, was adequate to maintain the low density porous nature of the formulation and prevent recrystallization. The ability to supersaturate SLF containing 0.02% DPPC and the presence of amorphous drug has been shown to increase both the Cmax and Tmax in the blood and lungs when compared to crystalline drug formulations dosed to ICR mice (20), and may be instrumental in improving bioavailability in humans.

To determine the effect of varying methods of degradation, a battery of accelerated degradation tests were performed. At absorbance measurements taken between 215 nm and 300 nm, all major degradation peaks showed the highest absorbance at 215 nm, allowing for detection of degradants and TAC at the same wavelength. Chromatographic results of acidic, alkaline, oxidative, and thermal degradation can be seen in Figure 3.3 and Table 1. TAC was susceptible to both alkaline and acidic hydrolysis, while thermal and oxidative degradation procedures had less effect. Based on chromatographic results of samples stored for up to 3 months, the manufactured drug product showed less than 0.2% degradants at each time point. Assuming that the potential human dose of TAC in this drug product is 6 mg once daily, this drug product is below the thresholds for identification (0.5% degradants) and qualification (1.0% degradants) according International Conference of Harmonisation (ICH) guidelines, and only requires that degradants be reported (28). Measurements of drug purity and potency

within the drug product were measured by HPLC analysis and are displayed in Table 2. Percent potency was calculated assuming that the theoretical potency of TAC was 50% of the formulation weight.

Organic solvent remaining after lyophilization is regulated by the FDA in drug products based on a toxicity classification system to ensure patient safety. Residual solvent may also accelerate or contribute to product physical instability due to its solubilizing effect on the active ingredient. Testing for residual solvent was conducted for quantification of ACN that may remain in the drug product after the URF production process. Based on trends in peak height and a chromatograph noise height of 500 counts, the detection limit (based on 3:1 signal to noise ratio) of this system was approximately 0.1 μL/mL for detection of ACN in DMSO. The limit of quantitation (LOQ) for this method based on a 10:1 signal to noise ratio is approximately 0.25 μL/mL for detection of ACN in DMSO. According to ICH guidelines, 410 ppm of ACN is the allowable concentration in a 10 g drug product (29). While our drug product is approximately 5 g after reconstitution with 5 mL saline, we assumed 410 ppm to be the maximum allowable concentration of ACN residual in TAC powder for reconstitution. As shown in Figure 3.4, no detectable amount of ACN was measured when a dose of 50 mg TAC powder for reconstitution was dissolved in 1 mL DMSO. It can therefore be determined that this drug product is below the maximum allowable ACN concentration, even at levels well beyond the anticipated human dose.

3.3.2 In vivo tolerability

To determine the amount of TAC present in the lungs and corresponding systemic concentrations, blood and lung tissue levels were analyzed by ELISA and LC/MS. After 28 consecutive daily nebulized doses, analysis of lung tissue and whole blood revealed

peak concentrations of 1758.7 ± 80.0 ng/g and 10.1 ± 1.4 ng/mL, respectively. Figure 3.5 shows the relationship between lung and blood concentrations at 1, 24, and 168 hrs after the final dose was administered. In a separate single dose study, rats exposed to the same nebulized dose (6.4 mg TAC colloidal dispersion) showed 1 hr tissue and blood levels of 294.8 ng/g and 4.9 ng/mL, respectively (study not yet published). These results demonstrate that there is substantial drug accumulation in the lungs after multiple pulmonary doses, reaching nearly 6 times single dose levels after 28 consecutive doses. It is most likely that accumulation of TAC is a result of the lipophilic nature of the molecule. TAC, with a Log P of 3.8, shows a strong preference for lipid solubilization, while CSA has a Log P of 4.3 (30). With a similar lipophilic nature and molecular weight, it might be expected TAC and CSA would behave similarly in vivo, binding to proteins and lipid membranes. Multiple studies have been conducted to evaluate the efficacy and pharmacokinetics of aerosolized CSA, as mentioned above. Similar to our findings, investigators have determined that cyclosporine exhibits substantial lung retention, having a mean terminal half life of 40.7 hr after aerosolization compared to 6.5 hr with intravenous administration (31). Rationale for lung retention has been attributed in part to two major mechanisms. First, drug interaction with phospholipids (32) and proteins (33) in pulmonary fluid as well as drug association with the pulmonary epithelial have been demonstrated and are believed to be a significant cause in pulmonary drug retention. Additionally, hydrophobic molecules such as CSA (34) and itraconazole (35) in the lung have been shown to experience macrophage uptake, where prolonged release and/or transport to bronchial-associated lymphoid tissue (BALT) may occur. Monitoring of TAC association with a common alveolar protein, surfactant protein A, has been accomplished in vitro by Canadas and coworkers. By derivatizing TAC with a fluorescing moiety, the equilibrium association constant and Gibbs binding free energy of

TAC to an alveolar lipoprotein were determined. Results showed that, like CSA, TAC preferentially binds to proteins in the alveolar fluid (33).

By providing high local concentrations and maintaining systemic levels well below those known to cause adverse side effects, inhaled TAC colloidal dispersion represents an improvement on current therapies for lung transplant recipients. Another potential application of inhaled TAC could be for supplementary dosing to assist with patients showing poor or highly variable gastrointestinal absorption. Administration of large immunosuppressive doses to the lungs may be able to contribute to maintenance of therapeutic blood levels by acting as a biological depot, resulting in slow release from lung tissue, reducing oral dosing requirements, and limiting the impact of poor intestinal absorption. This could be particularly useful in patients showing poor oral bioavailability, such as in cystic fibrosis populations, where the permeability of the intestinal wall is reduced (36). Investigation into the ability of the lungs to serve as a potential drug reservoir for sustained release will be investigated in future studies.

Comparison of CBC and serum chemistry results of rodents dosed for 28 consecutive days with TAC colloidal dispersion to those administered aerosolized normal saline showed no clinically significant difference for both 1 day and 1 week after the final dose (Table 3). While mean eosinophil count of the group sacrificed one week after the final dose did show significant difference (0.5% vs. 1.7%; $p < 0.01$) from the saline dosed group, this is still within the normal range (37). More importantly, the value was not significantly different after 28 days of treatment with inhaled tacrolimus and histological evaluation of the lungs revealed no increase in eosinophils for either treatment group. In fact, no individual animal in this study showed eosinophil levels higher that 196 cells/μL. Decreased platelet count may be due to partial clotting of samples and/or analysis of samples at room temperature rather than 37°C, which has been reported to cause

clumping in EDTA-anticoagulated blood (38). While TAC has shown to cause cases of diabetes mellitus and hyperglycemia, in our study hyperglycemia was also noted in control rats at statistically similar levels. The increased level of blood glucose can be attributed to the use of high levels of isoflurane which both decreases glucose clearance and increases glucose production (39).

Normal liver and kidney function were determined by serum chemistry analysis. The hepatic panel, seen in Table 4, while having no difference between groups, showed elevated aspirate amino transferase and alanine amino transferase levels for several potential reasons. Hypoxia after animal euthanasia, red blood cell hemolysis (40), and liver damage (41) caused by excessive isoflurane inhalation during sacrifice all could have contributed to these increased transferase levels. Significant differences were observed in creatinine and albumin levels; however, both showed improved kidney function relative to control. Change in serum creatinine was assumed clinically insignificant, since normal maintenance therapy with TAC is often associated with increased serum creatinine due to nephrotoxicity (42). Interestingly, increased albumin levels have been reported in the treatment of lupus nephritis with low doses of oral TAC (43). In one study, TAC trough levels of 4-6 ng/mL reversed hypoalbuminaemia in 5 of 6 patients over two years of therapy (44).

Evaluation of lung, liver, spleen, and kidney tissues in both experimental and control groups prepared by H&E stain showed no evidence of inflammation, cell lysis, or histologic lesions. Nephrotoxicity, one of the most common occurring side effects of TAC, is defined as interstitial lesions, vasoconstriction, and fibrosis of renal tissue (45). Over expression of transforming growth factor (TGF)-β α –smooth muscle actin leads to an overproduction of fibrous tissue, normally associated with wound healing and vasoconstriction (46). Calcineurin inhibitor-induced nephrotoxicity is

defined structurally as any occurrence of lesions within the fixed specimen. Nephrotoxicity can be diagnosed pathologically when tissue biopsy shows evidence of progressive arteriolar hyalinosis, striped cortical fibrosis, or severe tubular microcalcification (47). In our study, histological examinations of the kidneys showed no evidence of nephrotoxicity, and were in distinguishable from controls.

In addition to H&E staining, the lungs were stained with CD68 and PAS for determination of alveolar macrophage and monocyte presence and airway mucus production, respectively. Figure 3.6 displays lung sections representative of those evaluated in for each stain. Results in both cases showed no difference to control slides analyzed, meaning inhaled TAC did not cause increased macrophage, monocyte, or mucus production, which may commonly be associated with pulmonary irritation. These results demonstrate that inhaled TAC not only is safe in rodents, but may also cause minimal-to-no irritation. Considering the only excipient included in the formulation is lactose, an excipient generally recognized as safe for inhalation, one would not expect considerable toxicity in the lung unless it was drug related. As mentioned previously, inhaled CSA, aerosolized by solubilizing CSA in propylene glycol, has been successful in clinical testing in humans (7); however, it has produced upper airway irritation in a portion of patients, most likely due to the hygroscopicity of propylene glycol.

3.4 CONCLUSION

It has been successfully demonstrated that TAC colloidal dispersion dosed once daily for 28 days at clinically relevant levels does not induce signs of blood or tissue toxicity. Multiple doses showed some lung accumulation; however, systemic levels remained lower than those seen in normal oral dosing. No significant changes were seen in hepatic and renal function panels and lung tissue showed no signs of damage or

inflammation. Furthermore, TAC powder for reconstitution proved to maintain its physical and chemical properties throughout the 3 month testing period

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Chapter 4: Evaluation of Tacrolimus Dispersion for Nebulization in an Ovalbumin-induced Asthma Model

4.1 INTRODUCTION

Asthma is a reversible respiratory disease characterized by epithelial inflammation, increased mucus production, and constriction of the airways. Effecting over 300 million people worldwide (1), asthma is a common disease that represents a significant burden to international heath care. Anti-inflammatory and bronchodilator therapy, provided by inhaled corticosterioids (IC) and β2- agonists, is commonly used to treat the symptoms of asthma, and is effective in most patients. As a first line therapy for treatment of asthma, use of IC is increasing among all age groups. As a consequence of frequent use, many patients develop partial or full resistance to corticosteroids, resulting in a need for increased dosage or alternative therapy. These patients constitute approximately 5 to 10 % of all asthmatics account for nearly 50% asthma related health care costs (2). Increased IC dosage is often associated with unwanted complications, particularly in children (3), and can lead to adverse side effects such as adrenal insufficiency, glucose intolerance, skin thinning, decreased bone density, and potential growth suppression (4). As a result of these complications and developed tolerance to corticosteroid-based asthma therapy, alternative anti-inflammatory pharmaceuticals have been investigated.

Immunomodulaing therapies using both small molecule pharmaceuticals and biopharmaceutical have been investigated for treatment of corticosteroid resistance (CR) in asthma; however none of been generally accepted the standard therapy.

Biopharmaceuticals have been developed for treatment of severe or CR asthma and specifically designed to bind with and block immune-signaling cytokines and antibodies. Specifically, tumor necrosis factor alpha (TNF-α) has been targeted due to its role in the Th-1 immune response, which is unresponsive to corticosteroids and is thought to be a driver of severe asthma. Infliximab and etanercept have been developed and investigated for their capability of decreasing unbound TNF-α in blood and lung tissue, although overall efficacy is still uncertain (5). Immunoglobulin E (IgE) blocking antibody (omalizumab) has also been investigated as a biopharmaceutical therapeutic for severe asthma. Trials have shown clinical improvement in approximately two-thirds of patients who received this treatment for 12 to 16 weeks along with IC and β2- agonists, suggesting that omalizumab may be effective for some patients as an add-on therapy (6). Some of the more frequently investigated non-biologic alternatives to β2-agonists and IC include methotrexate, gold, and cyclosporine; all of which have been given orally. The efficacy of these therapies has been determined in reduction of inflammation and corticosteroid dependence in multiple clinical trials (7).

4.1.1 Calcineurin inhibitors in asthma trials

Calcineurin inhibitors are immunosuppressive drugs that disrupt early activation and signaling on the immune response and are frequently used in oral maintenance therapy for transplant recipients. Cyclosporine, a well characterized calcineurin inhibitor, has been investigated for treatment of asthma due to its ability inhibit a broad range of interleukins and other signaling factors that lead to T-cell activation. In clinical trials, cyclosporine has been shown to reduce corticosteroid dependency while improving lung function in cases of severe asthma (8, 9). Limitation of the adverse side effects associated with corticosteroid therapy may be achieved with calcenurin inhibitors;

although, the untoward side effects of these immunosuppressants have also been well documented (10, 11). To minimize potential systemic effects, inhaled cyclosporine has been investigated in lung transplant patients (12) as well as in animal asthma models (13). Tacrolimus, a calcineurin inhibitor up to 100 times more potent that cyclosporine (14), has more recently been accepted as a more effective therapy for transplant patients, suggesting it may also show improved outcomes for treatment of asthma.

4.1.2 Efficacy in asthma models

Initial investigation into the application of tacrolimus to asthma therapy has mostly limited to animal models. In early pre-clinical testing, tacrolimus was investigated as an immunosuppressive agent to be given orally for the suppression of lung eosinophilia, a common indication of airway inflammation. Three different dosing regimens were used in ovalbumin-sensitized Dunkin-Hartley guinea-pigs to determine if oral tacrolimus could effectively prevent eosinophil migration into the airway. Results showed that once-daily oral dosing for three days after sensitization and three days after challenge significantly reduced eosinophils count in bronchoalveolar lavage (BAL) fluid compared to controls (15). Surprisingly, when the immunosuppressive regimen was dosed for three days directly before antigen challenge, no reduction in eosinophiliar migration was seen in any of the drugs tested (tacrolimus, cyclosporine, cyclophosphamide). Findings in this study suggest that tacrolimus may play more of a role in disrupting T-cell response to initial antigen sensitization as well as late stage inflammation characterized by eosinophils recruitment. This may suggest that tacrolimus is better suited to treat severe refractory asthma rather than acute asthma. Additional research into the reduction of airway inflammation through administration of subcutaneous tacrolimus was performed by Lapa and coworkers. By administration of

0.5 mg/kg of tacrolimus 1 hour before and 5 hours after ovalbumin challenge, eosinophil migration into the airway/bronchial tissue as well as bronchial hyperactivity to acetylcholine challenge was prevented (16).

More recently, studies of tacrolimus intended for localized administration to the lungs has been developed and tested in antigen-sensitized animal models. Morishita and associates have investigated whether local delivery of tacrolimus to an asthma-induced airway will significantly inhibit inflammation. In albumin sensitized Hartley guinea pigs, it was shown that pMDI delivery of 150μg of tacrolimus given before antigen challenge was comparable to 1 mg/kg oral tacrolimus in the reduction of airway inflammation (17). Interestingly, peak lung and blood drug concentrations were 8 and 7 times lower for the inhaled tacrolimus than in oral dosing, showing potential to localize the drug effect without systemic effects. In this model, tacrolimus significantly reduced IL-5 levels in lung tissue and, correspondingly, airway eosinophilia. While anit-IL-5 antibody therapy has been proven somewhat clinically ineffective (18), tacrolimus shows promise to block multiple pathways of asthmatic inflammation through inhibition of multiple cytokines (IL-2, IFN-γ, IL-4).

In excised human lung tissue, tacrolimus was investigated and compared to dexamethasone to determine the extent of cytokine inhibition, mRNA expression, and chemical mediator suppression of each drug (19). Both drugs showed evidence of IL-5 and TNF-α inhibition, which were most likely to be produced by mast cells since the ex vivo study limited the amount of eosinophils and lymphocytes available from blood for cytokine production. These findings were expected and are well documented in literature for both steroid and immunosuppressive agents. More significant findings were shown in investigation of the chemical mediators of allergy, histamine and leukotriene E4. As shown in Figure 2, tacrolimus significantly inhibited release of histamine and leukotriene

E4, while dexamethasone did not. The exact mechanism of mediator inhibition is not known; however, this inhibition has been reported in literature previously (20). It is hypothesized that tacrolimus interferes with the stem cell factor regulation of mature mast cells, effectively preventing histamine release. This same study also found that while tacrolimus inhibits histamine excretion, it actually promotes mast cell differentiation in vivo. In addition to affecting T-lymphocytes and mast cells, tacrolimus has also been found to inhibit eosinophils degranulation (21).

4.1.3 Study rationale

In this study we aim to investigate the efficacy of a novel inhaled tacrolimus formulation in a widely accepted asthma-induced mouse model. We hypothesize that prophylactic dosing of tacrolimus dispersion for nebulization to asthma induced-mice will show significant reduction of lung inflammation compared to those given no treatment. Inflammation and chemical signaling were measured by white blood cell infiltration into the airways/tissue and cytokine concentration in lung fluid. Additionally, prophylactic treatment with an inhaled corticosteroid, dexamethasone, was performed for validation of our model for the evaluation of prophylactic therapeutics.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Tacrolimus was purchased from Haorui Pharma-Chem, Inc (Edison, NJ). Other chemicals used for sensitization and dosing included lactose monohydrate, egg albumin (ovalbumin) spray dried powder, and dexamethasone sodium phosphate, all purchased from Fisher Scientific (Fair Lawn, NJ). Aluminum hydroxide gel adjuvant

(Alhydrogel 1.3%) was obtained from Accurate Chemical (Westbury, NY). Normal saline (0.9% sodium chloride inj, USP) was purchased from Hospira, Inc (Lake Forest, IL). Hepairn Sodium (10,000 IU/mL) was purchased form Baxter Healthcare (Deerfield, IL). Formalin solution (10%) was purchased from Sigma-Aldrich (St. Louis, MO). High Performatnce Liquid Chromatography (HPLC) grade acetonitrile, zinc sulfate heptahydrate, and barium hydroxide (0.3N) were also purchased form Fisher Scientific (Fair Lawn, NJ).

4.2.2 Asthma model and dosing regimen

An ovalbumin-induced asthma model was developed using female BALB/c mice, based on previously proven models (22, 23). A 28 day model was developed, where sensitization took place over 25 days, and challenge (induction of inflammatory response) occurred over the final 3 days. Animals were euthanized on day 29 for analysis. A total of 37 mice were used and broken down into 4 groups: Negative control (5 mice), Positive control (14 mice), Tacrolimus dose (12 mice), and Dexamethasone dose (6 mice).

Sensitization occurred on day 0 and day 14 where 0.1 mL intraperitoneal (IP) injections of 0.025 % w/v OVA were administered to each animal. Negative control group received 0.1 mL IP normal saline (NS). IP injections were prepared by combining 2.5 mg OVA with 10 mL alhydrogel in a 15 mL sterile centrifuge tube and mixing (300 rpm) at room temperature for 1 hour. On days 26, 27, and 28, mice were challenged with aerosolized ovalbumin (OVA) solution after prophylactic aerosol dosing. For challenge, a 10 mg/mL OVA solution was prepared by adding 300 mg OVA to 30 mL NS. All except the negative control group were challenged with 20 minutes of nebulized OVA solution on three consecutive days (day 26, 27, and 28). The negative control group

received 20 minutes of aerosolized NS on the same days. Prophylactic dosing occurred 1 hour prior to each challenge and involved aerosolization of either tacrolimus dispersion for nebulization (TAC), dexamethasone solution (DEX), or NS. TAC was prepared by incorporation of 45.3 mg tacrolimus powder for dispersion, comprising tacrolimus and lactose (1:1) (24), into 3 mL NS by probe sonication with a Branson Sonifier 450 (Danbury CT) (output 6, 10% interval). The 3 mL dispersion was added to the nebulizer reservoir within 30 minutes of preparation and nebulized to completion. DEX was prepared by dissolving 5 mg dexamethasone sodium phosphate in 50 mL NS, mixing at 300 rpm for 1 hour, and storing at 4°C until use. For dosing, 5 mL DEX was added to the nebulizer reservoir and nebulized to completion. Positive and negative control groups received 5 mL NS for nebulization instead of prophylactic treatment. Twenty-four hours after completion of the third challenge, animals were euthanized by isoflurane inhalation followed by cardiac puncture.

All aerosol dosing was conducted in a 6 port nose-only dosing chamber (24) in accordance with an Institutional Animal Care and Use Committee (IACUC) approved protocol. Aerosols were produced by an Aeroneb® Pro (Aerogen, Galway, Ireland) vibrating mesh nebulizer and entered the dosing chamber at 1 L/min.

4.2.3 Determination of tacrolimus lung levels

Tacrolimus lung deposition was quantified by measuring the lung concentration of 4 mice after dosing 45.3 mg of tacrolimus dispersion for nebulization in 3 mL NS. Excised lung tissue from mice euthanized 1 hour after completion of nebulization was subjected to an extraction procedure from quantification of drug levels. The extraction procedure is detailed in Section 3.2.4.3. Briefly, lung tissue was injected with an internal standard and homogenized in NS. To facilitate protein separation from drug, 0.3N

barium hydroxide, 0.4N zinc sulfate, and acetonitrile (ACN) were added to the homogenate. The homogenate was centrifuged to collect the supernatant solution and remove precipitated protein. Samples were dried and reconstituted in acetonitrile. Liquid chromatography / mass spectrometry (LC/MS) was used to quantify tacrolimus in prepared samples. A Thermo Fisher Surveyor Plus HPLC system with PDA (Waltham, MA) was used in conjunction with a Thermo Fisher LTQ FT Ultra Hybrid Mass Spectrometer (Waltham, MA) for determination of tacrolimus extracted.

4.2.4 Complete blood count

After euthanasia, blood drawn from cardiac puncture was added to BD Microtainer® tubes with K2EDTA to prevent coagulation. Samples from each animal were submitted for analysis by IDEXX preclinical research services (Westbrook, ME) and run within 24 hours.

4.2.5 Differential WBC count of BALF

Bronchoalveolar lavage (BAL) was performed after euthanasia for analysis of cells and proteins present in pulmonary fluid and on the epithelial layer. Gavage tubes attached to 1 mL syringes were inserted into the trachea through a small incision and tied tightly with suture to create a water-tight seal. Phosphate-buffered saline (PBS) (0.8 mL) was instilled into the lungs and withdrawn slowly. Bronchoalveolar lavage fluid (BALF) was then added to a conical vial and centrifuged at 420 x g for 2 minutes. A 100 μL sample of supernatant was removed for cytokine analysis and replaced with an equal volume of PBS. Samples were shipped at

approximately 4°C to IDEXX preclinical research services (Westbrook, ME) for slide preparation and pathologist reviewed differential white blood cell (WBC) counts.

4.2.6 Cytokine analysis in BAL

Quantification of cytokines, specifically interleukins 3 (IL-3), 4 (IL-4), 5 (IL-5), 13 (IL-13), were determined using a custom mouse quantibody array (Raybiotech, Inc, Norcross, GA). Procedures were followed as directed in supplier protocol. A 16 well quantibody slide was used, 6 wells devoted to standards and 10 devoted to samples. Blocking of the slide was conducted by adding 100 μL of sample diluent into each well incubating for 30 minutes on an orbital shaker (200 rpm). After blocking, diluent was removed and replaced with standards and samples for 1 hour of incubation. The slide was then washed with supplied wash buffer after which the detection antibody cocktail was reconstituted and added to each well (80 μL). Removal of antibody cocktail was followed by washing. Cy3 equivalent dye-conjugated streptavidin was then added (80 μL) to provide a fluorescing signal. The slide was again washed and read using a GenePix® 4000B scanner and GenePix® Pro software (MDS Analytical Technologies Inc., Sunnyvale, CA).

4.2.7 Pathological tissue evaluation

After BAL, while the gavage tube was still inserted in the trachea, approximately 1 mL of 10 % formalin was instilled, fully inflating the lungs. The trachea was then tied off, and the heart-lung block was excised from the thoracic cavity and submerged in formalin. Tissues were shipped to IDEXX preclinical research services (Westbrook, ME) where they were trimmed, processed, blocked, sectioned, and stained

with H&E. An animal pathologist reviewed all slides for inflammatory cell infiltrate and pulmonary lesions. Inflammation scores were assigned to each sample investigated based on mixed cell infiltration and were assigned a score of 0-4, where 0 shows no signs of inflammation and 4 is severe inflammation.

4.2.8 Statistical analysis

Pathological scoring, CBC, and BAL analysis were subject to statistical analysis to determine significant differences in data collected. One way ANOVA followed by a post hoc Tukey test (if necessary) performed using Minitab® Release 14 statistical software (Minitab Inc., State College, PA). Confidence intervals ranging from $p < 0.1$ to $p < 0.0001$ were used to determine significant differences.

4.3 RESULTS AND DISCUSSION

4.3.1 Model validation

Validation of the ovalbumin-induced asthma model used in this study was determined through pathological review of sectioned and stained lung tissue, BAL analysis, and CBC analysis of whole blood. Typically, lung inflammation in BALB/c mice is quantified by mixed inflammatory cell infiltration, specifically, eosinophilia in BALF (25). Inflammation scores of positive and negative control mice are shown in Table 4.1. A marked difference is seen between these two groups since no inflammation was induced by ovalbumin, resulting in no detection of inflammation the negative control model after blinded pathological review. Infiltrate was characterized by mononuclear cells (mainly lymphoplasmacytic cells) and granulocytes (primarily neutrophils). Interestingly, perivascular infiltration of inflammatory cells was marked in the lungs of

asthma induced positive control mice. This finding is supported by previous studies that have reported an increase in vascular permeability in ovalbumin challenged rodents for up to 24 hours after delivery of ovalbumin aerosol (26).

Inflammation on the epithelial surface of challenged mice was also determined through assessment of BALF. Figure 1 shows changes in inflammatory cells present, specifically lymphocytes and eosinophils ($p < 0.005$), after OVA induced inflammation. Eosinophilia, a key feature of atopic asthma, is often used as a primary indicator the disease.

Circulating white blood cell (WBC) levels are also typically indicative of an immunological response. Table 4.4 shows an elevated mean WBC count for animals receiving ovalbumin sensitization and challenge, confirming that mild systemic immune response has occurred. A sensitization period of 21 to 28 days is used in most ovalbumin induced animal models (22, 23), and is likely sufficient to elicit an immune response upon antigen presentation. It is critical that the sensitization period is long enough to allow for "IgE switch". This phenomenon describes a change initiated by chemical signals that promotes B cell production of the IgE isotype of immunoglobulin. Both IL-4 and IL-13 have been demonstrated to be significant factors in promoting IgE switch in B cells, where production of IgM, a non-allergenic immunoglobulin, transitions to production IgE (27). Having a high binding affinity for mast cells and basophils, IgE bound with its receptor, FcεRI, is know play a prominent role in the initial cascade of inflammatory cytokines and chemokines. This is evident in the effectiveness seen in anti-IgE therapy in severe asthmatics. IgE has also been demonstrated to contribute local regulation of refractory or severe asthma (28), suggesting that local inhibition of IgE may be of considerable importance. For these reasons, IL-4 and IL-13 are considered instrumental cytokines in proliferation of traditional asthma. Since the role of IgE is

critical to asthmatic inflammation, a full "IgE switch" is necessary to produce the maximum response in an animal model.

4.3.2 Tacrolimus prophylaxis

Determination of tacrolimus deposition in the lungs showed that a mean of 0.566 μg tacrolimus was present after single dose administration (Table 4.4). As previously mentioned, tacrolimus dosed both orally and pulmonarily, has been shown to inhibit expression of both Th1 and Th2 cytokines in animal models; however, only cytokines involved with the Th2 response are evaluated in this study. Figure 4.2b shows a significant reduction ($p < 0.05$) in IL-13 expression in animals receiving tacrolimus prophylaxis in comparison to those with no prophylactic therapy. The biological effects of IL-13 are similar to that of IL-4, since they share a common alpha-chain receptor for signal transduction. IL-13 has been shown to be primarily involved in the recruitment of eosinophils and synthesis of IgE, two of the main physiological signs of a Th2 response (29). While no statistical difference is seen in IL-4 and IL-5 expression in tacrolimus dosed and positive control animals due to the variability of the bioassay, data trends toward reduction in these to cytokines after tacrolimus administration. This trend is supported by prior studies showing reduction in BAL levels of IL-4 and IL-5 in tacrolimus dosed animals (17).

Observations of data pertaining to cytokine expression in BALF are supported by the corresponding mixed inflammatory cell counts observed. Although variability is high, a significant difference ($p < 0.1$) is seen between eosinophil presence in BALF form tacrolimus and positive control groups. As eosinophil recruitment is thought to be signaled by IL-5 expression, the reduction of both of these factors (as seen in Figures 1 and 2b) is evidence of reduction of Th2 medicated asthma with tacrolimus.

Additionally, lymphocytes present in BALF are also statistically reduced ($p < 0.05$) in tacrolimus dosed animals. Lymphocytes are most likely inhibited on the epithelial surface due to tacrolimus inhibition of a variety of cytokines including IL-4, IL-5, IL-8, and IL-13. As in the reduction of eosinophilia in pulmonary fluid, reduction of immunoregulating lymphocytes also indicates the effect of tacrolimus on diminishment of the immune response to a foreign antigen.

Surprisingly, inflammation scores assigned by an animal pathologist did not correspond to the reduction of inflammation seen in BALF. Both positive control and tacrolimus dosed animals showed lymhoplasmacytic and neutrophilic inflammation, particularly around the lung vasculature. Cyclosporine, being mechanistically similar to tacrolimus, has shown similar results in recent findings. It has been previously noted that cyclosporine reduces expression of inflammatory cytokines such as IL-2, IL-4, IL-5, and IFN-γ as well as eosinophil infiltration, although airway hyperresponsiveness and neutrophil accumulation are unaffected (30). It may be that while calceneruin inhibitors have a pronounced effect on the inflammatory cascade induced locally, they do not reduce the vascular permeability of bronchial blood vessels, allowing initial neutorphilic infiltration into challenged tissue. This may explain why pathological evaluation of lung tissue sections did not demonstrate a reduction in perivascular infiltration of inflammatory cells, while inflammatory cells on the lung epithelium, as seen in BAL, are reduced. It is uncertain whether calcineurin inhibitors prevent vascular leakage, although these results would suggest that it does not.

It is worth noting that a significant difference was seen between the two groups receiving tacrolimus (denoted by F and G in Table 4.2). This difference suggests that there was some variability of scoring between groups and that additional pathological review may be needed.

4.3.3 Dexamethasone prophylaxis

Confirmation of inflammation reduction (as determined through BAL differential) with prophylactic dosing of dexamethasone validated the use of this model for the evaluation of prophylactic therapies. Reduction of lymphocyte ($p < 0.1$) and eosinophil ($p < 0.1$) presence in BALF compared to positive controls was found to be statistically significant, as would be expected in inhaled corticosteroid therapy. While the mean eosinophil levels of tacrolimus are lower than that of dexamethasone, due to high variability in BALF samples, no difference can be stated. In a previous study evaluating the effect of dexamethasone and cyclosporine on eosinophil proliferation in the presence of IL-5, researchers found eosinophil survival was more greatly inhibited by dexamethasone, while degranulation was more inhibited by cyclosporine (31). These findings indicated that cyclosporine (and likely tacrolimus) may effectively block the degranulating effects of IL-5 on eosinophils; although the exact mechanism by which this occurs is unknown. In reference to our results, this may suggest that tacrolimus in the lungs may reduce eosinophil levels due to the reduction degranulation, signaling, and subsequent eosinophil recruitment. This may prove to have a greater inhibitory effect on inflammatory signal proliferation and late stage asthma. Alternatively, dexamethasone reduces eosinophils in the airway due to disruption IL-5 signaling that normally promotes cell survival, resulting in apoptosis. This suggests that dexamethasone could be more relevant to the inhibition of acute or early stage asthma.

Evaluation of cytokine expression after prophylactic dosing with dexamethasone showed no signification difference from levels in the positive control group. It is uncertain why dexamethasone was associated with an increase in IL-5 expression, as it

has been well established that this glucocorticoid diminishes the expression of IL-5 (32). Variability in bioanalytical testing is most likely the reason for this inconsistency.

Prior art and BALF cell counts would suggest that dexamethasone does reduce signs of inflammation, although inflammation scoring of dexamethasone dosed animals proved to show no statistical difference from animals receiving no treatment. The mean score is lower in dexamethasone dosed rats than in tacrolimus dosed rats, suggesting more effective reduction of deep tissue inflammation. Permeability of pulmonary vasculature has been shown by researchers to be reduced by administration of β2- agonist as well as corticosteroids (33), which may explain the reduction in perivascular inflammation. More samples are needed to determine if a statistical difference is present.

Systemic circulation of WBC after challenge shows interesting changes in animals dosed with dexamethasone. Total WBC is reduced in comparison to tacrolimus treatment; although, not with statistical significance. A significant difference was seen in an increase in circulating neutrophils in comparison of dexamethasone treated animals to negative control animals. Clinically, corticosteroid administration often results in a temporary increase in circulating neutrophils due to demargination. Corticosteroids act on non-circulating neutrophils in the margination pool as well as promote the egress of neutrophils from bone marrow (34).

4.3.4 Calcineurin inhibitor and corticosteroid synergy

Recently, the mechanisms of CR that develop over repeated dosing have been investigated. Findings have shown that expression of FK506 binding protein 51 (FKBP-51), an intracellular chaperone protein, is induced by the presence of corticosteroids (35). Interestingly, FKBP-51 has been shown to disrupt glucocoritcoid receptor (GR) complex interaction with corticosteroids, inhibiting its translocation to the

nucleus and serving as a negative feedback loop. This finding is supported by preclinical studies in non-human primates that have shown that excessive expression of FKBP-51 to reduce efficacy of corticosteroids (36).

FKBP is named for the molecule for which it was first discovered to bind, FK 506, which is more commonly known as tacrolimus. Tacrolimus has been shown to have an affinity for FKBP-51; although, it is thought to be lower than its affinity for FKBP-12 (37). Nevertheless, it is plausible that tacrolimus co-administration with a corticosteroid could enhance the activity of that corticosteroid through interference with the FKBP-51 negative feedback loop. This would allow more effective steroid-induced nuclear translocation of the GR, resulting in transcription modulation for immunosuppression. In addition to the synergistic mechanisms associated with FKBP-51, it seems that physiological effects of these drugs may be therapeutically complementarily.

Another potential synergy may exist between calcineurin inhibitors (tacrolimus) and corticosteroids through reduction on neutrophilic inflammation. Immediate reduction of eosinophil levels in the airways often results from corticosteroid therapy; however, its effect on neutrophilic infiltration, which is more characteristic of severe asthma and airway remodeling, is uncertain (38). Clinical studies have found that increased neutrophil presence in the sputum reduces the effect of corticosteroids therapy on eosinophilic airway inflammation (39). Cyclosporine has been shown to limit neutrophil chemotaxis (40) due to inhibition of its major signaling cytokine, IL-8. Tacrolimus has also been show to inhibit IL-8 (41), and can therefore be assumed to prevent neutrophil chemotaxis as well. By preventing recruitment of neutrophils into the lung airways, neutrophilic inflammation can be prevented and inflammatory cell resistance to corticosteroids may be reduced. Aside from tacrolimus, other macrolides (antibiotic such as clarithromycin) have been found reduced IL-8 and neutrophilic inflammation in

patients (42); however, these antibiotic macrolides do not offer the breadth of cytokine inhibition and inflammation reduction seen in calcineurin inhibitors.

4.4 CONCLUSION

In an ovalbumin-induced asthma model, inhaled tacrolimus has shown to reduce the Th2 response when given 1 hour prior to challenge. This conclusion is supported by noted reduction in inflammatory cytokines and cells present in the airway of BALB/c mice. Likewise, dexamethasone showed a decrease in airway inflammation as evident through reduction of epithelial inflammatory cells, validating this model for prophylactic evaluation. Future studies will be conducted to evaluate the potential synergistic therapy of tacrolimus and dexamethasone based on potential complementary modes of inflammation reduction and molecular binding. Additionally, testing of inhaled tacrolimus in neutrophilic asthma model is necessary to determine its effect on Th1 mediated asthma in vivo.

This study has confirmed that pulmonary administration of tacrolimus can effectively inhibit the Th2 immune response. As an immunosuppressive agent with capability to inhibit a broad range of cytokines, tacrolimus shows promise as a therapeutic agent for cases of asthma where corticosteroid therapy is no longer effective.

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Chapter 5: Respirable Low-Density Microparticles Formed *In Situ* from Aerosolized Brittle Matrices

5.1 INTRODUCTION

Dry powder inhalation has been established as a method for pulmonary delivery and as a viable and efficacious method for lung disease management. Important advancements including multidose capabilities, improved device design, and drug/carrier particle engineering have led to improvements in drug aerosolization efficiency and patient compliance. While setbacks in use of dry powder inhalers (DPI), for systemic drugs have been noted, as in inhaled insulin (1), many academic and industrial researchers continue to investigate the potential of the pulmonary route as a fast, noninvasive means of systemic therapy that bypasses first pass metabolism.

Nearly all currently marketed DPI products rely on carrier-based formulations where micronized drug particles adhere to a coarse carrier, typically lactose. The carrier is used to improve powder flow through ordered mixing and reduce electrostatic and van der Waals interactions between micronized drug particles. Inertial and aerodynamic forces provided by a combination of patient inspiration and device design subsequently deaggregate drug from carrier, allowing inhaled micronized drug particles to ultimately reach the deep lung. This formulation strategy is inexpensive and easily scalable; however, it can result in high dose variability and low efficiency, making it less than ideal for delivery of expensive biopharmaceuticals or potent drugs with a narrow therapeutic window. Typically, carrier-based DPIs can be expected to deliver only 10 to 35% of the emitted dose to the lower airways (2), while the majority of the dose fails to

deaggregate from the carrier particles and deposits in the mouth or on the oropharynx. Interest in delivery of more potent and expensive pharmaceuticals is leading to the investigation of more efficient and precise therapeutic formulations.

Recently, new formulation strategies have been designed for more effective aerosolization and flow-rate independent delivery. Large porous particles for inhalation were introduced by Edwards et al as a method to enable the delivery of geometrically large particles (20 µm) to the deep lung where sustained release of PLGA encapsulated drugs could occur (3). The fundamental concept behind this delivery strategy relies on the similar aerodynamic behavior of particles in the respirable range (1-5 µm) and large particles (>10 µm) with a low skeletal density (<0.4 g/mL). The relationship between aerodynamic (d_{ae}) and geometric (d_g) diameter is given in equation 1.

$$d_{ae} = d_g \left(\rho_s / X \right)^{0.5} \quad (1)$$

where ρ_s is the particle or skeletal density and X is the dynamic shape correction factor. The dynamic shape factor is necessary to calculate the equivalent aerodynamic diameter of particles with increased drag due to their nonspherical shape. Typically, dynamic shape factors range from 1 to 2, where 1 represents a perfectly spherical particle with a smooth surface and 2 represents a plate-like particle with a rough or irregular surface. Another aspect of aerodynamic behavior, particle slip, has also been show to effect the aerosolization of respirable particles, however, this factor need only be considered for particles less that 10 µm in diameter (2).

Several advantages have been proposed and demonstrated in the use of porous particles for lung delivery. In comparison to traditional DPIs which require the

generation of high flow rates for deaggregation, porous formulations are easily dispersed with less dependence on the air velocity. Increased deaggregation is thought to stem from limited surface contact and reduction of particle cohesion due to van der Waals forces. Additionally, elevated drag produced by high surface area and irregular surface morphology also contributes to improved powder dispersion. Once deposited in lower airway, particles with a geometric diameter between 1 and 5 µm are rapidly phagocytized by alveolar macrophages, 10% being cleared from the epithelial fluid within with first hour, and over 80% after 24 hours (4). Particle diameters less than 100 nm or more than 10 µm have shown evidence of greatly reduced macrophage clearance, enabling the possibility of sustained release drug therapy from the pulmonary epithelium. Porous particles in the deep lung, while too large for phagocytosis, also might be expected to be only partially submerged in the 7-70 nm thick layer of epithelial fluid lining the alveolar space (5), leaving only a portion of the particle available for dissolution. In contrast, nanoparticles are completely submerged and wetted in lung fluids after deposition and are available for partitioning into tissues and systemic circulation (6, 7). Furthermore, the high surface area and thin boundary layer facilitates rapid dissolution. (8-10)

Because nanoparticles are aerodynamically too small for deep lung delivery, they must be delivered in a microparticle carrier. Nanoparticles have been delivered by nebulization to the deep lungs as a dispersion in aqueous media (6, 11-15). Trojan particles have also enabled the delivery of nanosized drug through preformed hollow spherical nanoparticle aggregates produced by spray drying (16). Recently, open friable flocs of high aspect ratio drug particles, formed by thin film freezing, have been delivered to the deep lung with pressurized metered dose inhalers (7). The space filling high aspect ratio particles are templated by the hydrofluoroalkane droplets upon actuation to form

low-density particles with the proper aerodynamic diameter for deep lung delivery and high dosages.

Formulation technologies such as milling, spray drying, spray-freeze drying, supercritical fluid processing, and controlled aggregation have all been used in production of low-density, dry particles for inhalation (17-19). In this study, a new approach to low-density particle delivery is investigated using a cryogenic particle production method. In thin film freezing (TFF), a frozen solid solution is formed rapidly by dropping a solution directly onto a cryogenically cooled solid substrate. The rapid freezing prevents segregation and heterogeneity of the solutes (20). Solvent removal by lyophilization limits the mobility of the dissolved solute and results in a low-density matrix. This freezing technique has also been used to produce stable protein particles for inhalation (7) and was found to produce less denaturation than other cryogenic techniques due to the reduction of air exposure (21).

We hypothesize that highly respirable low-density microparticles can be produced by *in situ* shearing of aerosolized brittle matrices in a passive inhalation device. Powder geometries created by TFF are designed to have a lower skeletal density (< 0.05 g/cm^3) than those measured in previous studies (0.05 to 0.1 g/cm^3). The powders created by TFF are composed of large interwoven matrices rather than discrete particles (3). Additionally, the concept of *in situ* shearing of brittle matrices to produce respirable particles ~ 50 μm in diameter is novel relative to previous studies in which the particles for delivery are preformed. Given that the alveolar diameter has been shown to be 400 μm in human adults, these particles are sufficiently small for deep lung deposition (22). The high surface area of the brittle matrices, composed of sub-500 nm primary structures, produced by TFF enhances the dissolution rate, which favors high absorption and bioavailability (10, 20) Furthermore, clearance by alveolar macrophages before

dissolution may be reduced due to the large geometric size of deposited particles (3). Formulation, moisture sorption, and *in vitro* characterization techniques were also studied to optimize aerosolization and produce data not biased by particle bounce.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Alpha-lactose monohydrate, mannitol, and raffinose pentahydrate were purchased from Fisher Scientific (Fair Lawn, NJ). High Performance Liquid Chromatography (HPLC) grade acetonitrile and phosphoric acid (85%) were also purchased from Fisher Scientific. Tacrolimus anhydrous was purchased from Haroui Pharma-Chem (Edison, NJ).

5.2.2 Formulation preparation

TFF technology was employed for the production of dry powders. Briefly, a cosolvent mixture of acetonitrile (ACN) and water was used to dissolve tacrolimus and sugar excipient. Tacrolimus and lactose (TACLAC), tacrolimus and mannitol (TACMAN), tacrolimus and raffinose (TACRAF), and tacrolimus without a sugar excipient (TAC) were dissolved in the cosolvent solution. The ratio of tacrolimus to excipient was 1 to 1 and each solution prepared for TFF had a total solids concentration of 0.75% w/v. The solutions were rapidly frozen on a cryogenically cooled (< -50°C) stainless steel surface and then maintained in the frozen state in liquid nitrogen. A detailed description of the TFF process is given by Overhoff et al (23) and Engstrom et al (21). Solvents were sublimated by lyophilization using a VirTis Advantage Tray Lyophilizer (VirTis Company Inc., Gardiner, NY), leaving a drug and sugar solid

dispersion in dry low-density particles. Lyophilization was performed over 40 hours at pressures less than 200 mTorr while the shelf temperature was gradually ramped form -60°C to 25°C. Product was removed form the lyophilizer after dry N_2 was bled into the chamber to equilibrate to atmospheric pressure. Product was quickly covered in order to prevent ambient humidity from affecting the formulation. Powders were stored in a transparent vacuum desiccator at room temperature.

5.2.3 Powder density and compressibility

Bulk and tapped density of TFF produced powders were measured according to a method adapted from USP <616> method I using a Varian Tapped Density Tester (Varian, Palo Alto, CA). An adaptation was made due to the limited supply of powder for testing where a 100 mL graduated cylinder was replaced by a 5 mL graduated cylinder. Hausner ratio and Carr's (Compressibility) index were calculated for each formulation based on USP guidelines. Additionally, skeletal densities of dispersed powders were calculated based on measured aerodynamic and geometric diameter for comparison to measured density values. Calculations were performed according to equation 1, where the dynamic shape factor (X) was assumed to be 1.5 for all dispersed powders. Mass median aerodynamic diameter (MMAD) was determined based on all particles emitted from the device for these calculations.

5.2.4 Powder morphology and homogeneity

For qualitative determination of particle structure and morphology, scanning electron microscopy (SEM) was performed. A Hitachi S-5500 SEM (Hitachi High Technologies America, Inc., Pleasanton, CA) equipped with energy-dispersive X-ray

(EDX) was used at 10 kV accelerating voltage after sputter coating the specimen with silver for 30 seconds at vacuum. Determination of homogenous distribution of tacrolimus in lactose was determined by EDX through qualitative measurement of elemental nitrogen presence, which only occurs in drug molecule.

5.2.5 Geometric particle size analysis

Geometric diameter of TFF produced aerosolized and non-aerosolized powder was determined by low angle light scattering using a Malvern Spraytec® (Malvern, UK) outfitted with an inhalation cell and an induction port. A Handihaler® (Boeringher Ingelheim GmbH, Ingelheim am Rhein, Germany) containing a size 3 hypromellose (HPMC) capsule (Capsugel, Peapack, NJ) was secured to the mouth of the induction port by a molded silicone adapter. Aerosolization of powder was achieved at a flow rate 51 L/min, providing a 4 kPa pressure drop across the device. Data acquisition took place over 4 seconds and only when laser transmission dropped below 95%. Non-aerosolized powder diameter was measured by adding powders to the opening of the inhalation cell without the induction port and without air flow.

5.2.6 Aerodynamic particle size analysis

A Next Generation Pharmaceutical Impactor (NGI) (MSP Corp., Shoreview, MN) was used to determine aerodynamic properties of low-density microparticles. A Handihaler® containing size 3 capsules and approximately 3 mg of formulation was attached to the induction port by a molded silicone adapter. All tests, with the exception of those investigating the influence of gelatin capsules on aerodynamic diameter, were conducted with size 3 HPMC capsules. Aerosols were produced over 4 seconds at a flow

rate of 51 L/min. Stage cut size diameters were calculated as instructed by Marple et al to be 8.8, 4.9, 3.1, 1.8, 1.0, 0.6, 0.4, and 0.2 μm for stages 1 through 7 and micro-orifice collector (MOC), respectively (24). In most impaction tests run, collection surfaces were coated with 1% Tween 80 in ethanol, which is one of many coating materials recommended by the European Pharmaceutical Aerosol Group (EPAG) (25). Tween solution was applied to each collection surface (approx 1 mL) and allowed to dry for 1 hour. After aerosolization, collection of deposited powders was accomplished by rinsing with 2, 5, 10, and 2.5 mL mobile phase for the device, induction port, pre-separator (if used), and stages 1 – MOC, respectively. The pre-separator is designed to collect coarse particles (> 15 μm) before the enter the body of the NGI and was included only when coarse lactose is used. High performance liquid chromatography (HPLC) and a method for tacrolimus detection were used to quantify the collected drug from each rinsing (26). Samples were analyzed using a Waters 515 liquid chromatograph with a Waters 996 Photo Diode Array (Waters Corp., Milford, MA) at 215 nm. A Lichosphere RP C18 column 4 mm X 250 mm, 5 μm (Varian Corp. Lake Forest, CA) was used at 50 °C and a flow rate of 1.5 mL/min. Mobile phase was composed of ACN, water, and phosphoric acid at a ratio of 600 to 400 to 1.

Total emitted dose (TED) of each test was calculated as the percentage of dose emitted over total dose assayed. Fine particle fraction (FPF) and MMAD were calculated using Sigmaplot 2000 (Systat Software Inc, San Jose, CA) to fit a 3 parameter logistic curve to plotted data. MMAD and geometric standard deviation (GSD) were calculated based on drug deposition on stage 1 through MOC, while FPF was calculated based on TED and represent the percentage of particles with an aerodynamic diameter less than 5 μm.

5.2.7 Water sorption

Water sorption profiles were determined for brittle matrix powders manufactured by TFF using Dynamic Vapor Sorption (DVS-1) (Surface Measurement Systems Ltd, London, UK). For each formulation, glass sample cells were filled to capacity (0.5 mL) resulting in weights ranging from 5 to 30 mg, depending of particle density. Samples were dried with nitrogen gas until a baseline was established with less than 0.002 % change in dm/dt. Each formulation was run for a complete sorption/desorption cycle between 0 and 90% relative humidity (RH). Humidity was increased/decreased by 5% after equilibrium was reached, as determined by a dm/dt less than 0.002%. Sorption isotherms were calculated and plotted according to percent change in mass minus the initial dry formulation weight. DVS was also used to create a controlled humidity environment for powder dispersion to be tested using laser scatter. Humidities of 90, 50, 20, and 0% were exposed to powder formulations for 30 minutes in succession. Equilibrium was assumed after 30 minutes, and an aliquot of powder was removed for testing. All testing began with 90% humidity so that skeletal density changes due to hygroscopicity would be applied to subsequent samples taken at 50, 20, and 0% RH.

5.3 RESULTS AND DISCUSSION

5.3.1 Influence of testing conditions

Prior to characterization of TFF produced brittle matrices, methods of analysis, specifically NGI testing, were evaluated. For determination of aerodynamic diameter, many researchers have noted that NGI testing conditions and parameters can greatly influence results, the extent of which is often dependant on formulation properties (27). For example, liquid aerosols have been shown to experience droplet evaporation during

testing, causing a skewing of data toward aerosols with smaller MMADs and larger FPFs. The dry environment of impaction equipment is most likely not predictive of aerosol performance *in vivo* since physiological relative humidites in the airways are near 100%. Adjustments for humidity have been made by cooling NGI equipment prior to use, resulting in a device internal RH of approximately 100% (28). Testing of dry aerosols produced by pMDI and DPI devices may also show bias in some cases due to particle bounce and re-entrainment. This behavior has been addressed by several authors, the majority of which recommend correction by coating of collection surfaces (27, 29). While no guidelines have been adopted by the USP for collection plate coating, the EPAG has recommended coatings of Brij, silicone, Tween, among others, to create a thin film to cause impacted particles to adhere after deposition (25).

The influence of particle bounce on NGI characterization of low-density powders has not been fully investigated, and was therefore considered before evaluation of low-density microparticles. As seen in Table 1, a substantial increase in MMAD was seen when a film coating was applied to stages 1 – 7 and MOC. During investigation of beclomethasone dipropionate aerosols of unit density, Kamiya et al showed an increase in MMAD of 1.06 μm after NGI collection plates were surface coated (30). A more exaggerated difference was seen in the low-density particles tested in this study showing an MMAD increase of 3.1 μm after coating. Fine particle fraction was also affected by surface coating, decreasing almost two fold when tested on coated surfaces. It could be assumed that highly porous particles with low density are not only subject to particle bounce, but that particle fracture and re-entrainment may also occur after plate impaction. Similar fracturing effects may also explain reduction in MMAD when a pre-separator was included (Table 1). Although addition of a pre-separator would be expected to affect MMAD based on reduced deposition on stage 1, the skewing of deposition toward plates

4 through 6 suggests that particle fracture in the pre-separator occurred (Figure 1). Given the potential for MMAD biasing and the fact that no coarse carrier particles are included in this DPI formulation, pre-separators should be excluded when characterizing brittle matrices for aerosolization.

5.3.2 Investigation of formulation strategy

TFF technology produces low-density pharmaceutical matrices, often containing amorphous drug, stabilized with high T_g excipients (20). In previous reports, TFF has been used as a particle engineering technology to enhance the aqueous solubility of poorly water soluble drugs for oral and pulmonary applications (7, 31). Through stabilization of amorphous drug morphologies with glassy excipients, inclusion of hydrophilic materials, and increased surface area, TFF manufactured powders have been shown to offer improvements in wetting, dissolution rates, and solubility, leading ultimately to increased bioavailability (12, 32-34). Given the desirable attributes of these powders and the efficiency of low-density powders for deep lung delivery, we hypothesized that these particles would result in superior aerosol performance relative to previously researched porous particles made by traditional manufacturing techniques. The primary and bulk structure of aerosolized brittle matrices of tacrolimus and lactose are shown in SEM images of Figure 2. In this formulation, drug and excipient are present in a one-to-one ratio in a solid dispersion. SEM samples analyzed by EDX reveal a homogeneous dispersion of tacrolimus and lactose, indicated by the presence of nitrogen (Figure 3b). Other studies have produced amorphous powders with TFF and have shown through x-ray diffraction (XRD) patterns and differential scanning calorimetry (DSC) that these dispersions often form solid solutions (34).

For effective delivery of respirable low-density microparticles, a passive inhalation device with the ability to produce high shear velocities is required. Fortunately, most device designs already require turbulent, high shear airflow to provide adequate force for the separation of micronized drug from carrier lactose. The Handihaler®, a single dose capsule-based DPI, was chosen for aerosolization of brittle matrices in this study. Through a patient induced pressure drop, contents of a size 3 capsule within the device are released by flow within and around the capsule. Prior to discussion of further formulation considerations, aerosol performance dependence on capsule composition is first investigated. HPMC and gelatin capsules were studied for their influence on the aerodynamic performance of powders emitted. Significantly different behavior was observed. Interestingly, Table 2 shows HPMC capsules produced a significant improvement ($P < 0.05$) in FPF over that of gelatin capsules while MMAD was unchanged. We hypothesize that shape and area of the puncture hole created could influence the velocity/turbidity of air entering and leaving the capsule. In previous reports of puncture shape of gelatin and HPMC capsules, it was concluded that more irregularly shaped holes were formed in the less brittle HPMC capsules, relative to gelatin (35). For delivery of this formulation, a smaller, non-spherical puncture may provide a greater shear force than a large spherical opening, imparting for fracture of friable matrices. Other non-aerodynamic advantages of powders released from HPMC capsules include low moisture content and increased stability at elevated humidity (36).

Determination of friability of brittle matrix formulations was performed by comparing geometric particle distribution of low-density particles emitted from the DPI device with that of “bulk” or non-aerosolized matrices. The effect of shearing induced by the Handihaler® was substantial as indicated by the difference between the volume moment mean ($d_{4,3}$) of bulk (502.4 μm) and DPI emitted (62.0μm) particles (Figure 4).

The volume moment mean is a numerical representation of the "center of gravity" of a volumetric distribution, also known as the De Brouckere mean diameter (37). Because particle fracture is vital to the aerodynamic performance of these particles, excipient selection focusing on material properties such as strength, brittleness, and hygroscopicity is critical. The ability to fracture the bulk particles with air flow is consistent with the fracture of large open friable flocs of similar particles produced by TFF, in which shear was produced by a hydrofluoralkane in a pMDI (7). In each case, the shear produces particles with proper aerodynamic and geometric diameters to achieve high fine particle fractions. A major difference for the pMDI approach is that the particles collapse as the HFA droplets evaporate. An additional caveat to formulation of dry powder for inhalation is that these excipients be nontoxic and non-irritating for delivery to the lungs or otherwise generally recognized as safe (GRAS) by the FDA.

5.3.3 Pharmaceutical sugars in aerosolized brittle matrices

The influence of pharmaceutical sugars on aerosol performance was determined by measurement of both geometric and aerodynamic properties. In this pulmonary delivery platform, the fundamental principle for producing highly respirable microparticles relies on the brittle fracture of ultra low-density matrices to create small diameter particles of the same structure and density. Accordingly, pharmaceutical materials shown to experience brittle fracture under applied stress were chosen, such as those used in direct compression (DC) tabletting. Sacchirides used for DC are more likely to experience brittle fracture than ductile cellulose excipients, and are more appropriate for our application. In addition, some sacchirides are established as being non-irritating in the lungs. Two sacchirides, α-lactose and raffinose, were selected based on their brittle properties; however, the ability to induce brittle fracture with a passive

DPI device had not been determined. Mannitol, a less hygroscopic sugar alcohol, was also selected for evaluation as an excipient in brittle matrix powders. After production, initial visual observations of unpackaged product showed that the skeletal structure of TACMAN and TAC were less susceptible to ambient humidity than other formulations.

Aerodynamic evaluation of emitted low-density microparticles on a stage coated NGI showed elevated TED and FPF when compared to traditional dry powder inhalation formulations (2). Initial testing of newly prepared formulation revealed TACLAC and TACRAF as the most efficiently performing aerosols, with FPF of 70.3 and 63.5%, respectively (Table 3). Distribution of deposition on within the NGI, shown in Figure 5a reveals a lower stage deposition of TACLAC and TACRAF in comparison with the other formulations. Assuming that all formulations have similar density, it could be concluded that increased particle fracture of particles containing anhydrous α-lactose and anhydrous raffinose resulted in improved aerodynamic properties. Although some drawbacks to anhydrous material exists (as will be discussed), complete water removal from sacchirides often results in an significant increase in friability and brittleness (38, 39). Specifically, anhydrous raffinose is noted for its friability and has been determined to be the “most fragile” pharmaceutical sugar (38). It is interesting to note that the more brittle, anhydrous form of raffinose is also amorphous, contrasting with the general conception that amorphous sugars are more ductile. Differing from raffinose, anhydrous α-lactose is similar to others excipients in that the amorphous form is commonly less brittle than the crystalline. High TED for all formulated powders is indicative of the reduced surface cohesion of low-density powders, normally caused by van der Waals, capillary, and electrostatic forces in traditional formulations; although, further analysis shows that these forces do still play a role in particle dispersion.

Effect of RH on the aerosols comprising low-density microparticles was studied to determine sensitivity to water sorption. Figure 5 and Table 3 show a comparison of NGI tested formulation stored at 50%RH and results from initial tests. Humidity had an inhibitory effect on the performance of TACLAC, most likely due to increased plasticity of the brittle matrix. TACMAN proved to benefit from additional moisture, as shown by an increase in FPF, perhaps due to reduction in electrostatic charging. Figure 6b shows the bimodal distribution indicative of electrostatic adhesion of TACMAN at low RH. It can also be seen that TED decreased slightly in every 50% RH formulation, which could be expected due added formulation adhesion to the capsule wall in the presence of moisture. Water sorption to powder surfaces can both improve and hinder aerosol dispensability. Previous reports have shown that dry powder formulations stored at approximately 60% RH maximize the drug FPF (40). In general, humidities > 60% result in capillary forces predominating, while electrostatic charge remains low. Relative humidities < 60% will cause elevated electrostatic adhesion of powders due to the lack of moisture-induced charge dissipation. For brittle matrices, presuming they are amorphous, the plasticizing effect of water must also be considered. Amorphous materials are particularly susceptible to water plasticization, as is the case for anhydrous lactose and raffinose, which will result in reduced brittle fracture and could lead to increased particle density due collapse of the matrix structure (39).

5.3.4 Influence of moisture sorption

Based on differences in aerosol characteristics between initially tested and powder tested after storage at ambient conditions, moisture sorption may play a key role in the respiration of low-density microparticles. Determination of moisture sorption of each formulation at RH between 0 and 90 percent was determined gravimetrically by DVS.

Sorption and desorption isotherms were determined after one cycle and shown in Figure 7. As might be expected, hydrophilic materials lactose and raffinose showed high moisture sorption, particularly at RH above 70 %. Previous investigators have determined that raffinose samples with more than 17.9% weight gain due to moisture are capable of forming the pentahydrous form, although no reduction in mass loss due to crystallization is apparent in sorption analysis. Unlike the rapid crystallization seen in lactose, it has been reported that full crystallization of raffinose takes up to 30 hours to occur at elevated humidity (41), so crystallization would not be apparent at each step. Slow moisture uptake by raffinose was evident during sorption testing of TACRAF using the method described in this study, since slow water absorption (and corresponding slow baseline stabilization) meant long cycle times. The time required for raffinose to complete sorption/desorption cycle was 75 hours, while lactose only required 35 hours. This leads to the conclusion that both materials are very hydroscopic; although, anhydrous lactose adsorbs water much more readily.

Multiple cycle DVS of TACLAC also provided incite into morphological changes of the particles (Figure 8). While a mass of moisture sorbed typically increases with increasing humidity, beginning at approximately 55% RH and 5% w/w water sorption TACLAC began to undergo stints of moisture loss, which is indicative of crystallization (42). Crystallization during these three cycles is also made evident by the decreased water sorption capacity in cycles 2 and 3. It is apparent for DVS analysis that TACLAC, created as an amorphous powder, is unstable at ambient humidity (50%) and requires packaging with dry gas. TACRAF, while able to absorb greater amounts of water, did not experience morphological changes due to moisture adsorption.

To determine the influence of moisture adsorption on aerosol production, volume distribution of each formulation at humidities from 0 to 90% were determined. Each

sample was exposed to 90% relative humidity before the appropriate humidity was applied so that moisture-induced density changes (matrix collapse) would not cause skewing of volumetric size distributions. All formulations experienced electrostatic charging at 0% RH, resulting in bimodal particle size distribution of each formulation (Figure 6). Similar moisture induced bimodal distribution was seen in TACLAC when stored at 90%RH; although, in this case cohesion could be assumed to be due to capillary forces of adsorbed water. Raffinose, being more hygroscopic than lactose, might be expected to show similar cohesion at high humidities; however, raffinose is known to incorporate water in crystal hydrates so much of the water is not present on the particle surface to induce cohesion (41). Increased brittleness resulting in smaller particle diameters of TACRAF is noted in Figure 6c due to formation of anhydrous/amorphous material at 0% RH. In dry conditions, previous studies have observed that hydrogen bonding between raffinose and water is replaced by bonding between neighboring raffinose molecules, leading to disorder in packing (or amorphous state) (41). Humidity influence on TACMAN (Figure 6b) and TAC (Figure 6d) proved to be quite similar, which is a reflection on their similar structural and morphological stability during moisture changes. While it seems only electrostatic adhesion had a significant impact on the particle distribution of TAC and TACMAN, TAC at 0% RH appears to undergo increased brittle fracture.

Although direct testing of material brittleness and friability was not conducted (as in brittle fracture index testing (43) an interesting correlation can be drawn between the $d_{4,3}$ DPI emitted microparticles and "bulk" powders from each formulation (Table 4). In agreement with previous findings, particle brittleness (as determined by ratio of $d_{4,3}$ sheared to unsheared) of lactose and raffinose formulations proved to be greater than those of mannitol and tacrolimus.

Further investigation into anti-cohesive and hydrophobic materials (i.e. leucine, DPPC) may help limit water adsorption, decreasing cohesion at high humidity (44). Added aerodynamic improvements may also be imparted by promoting crystallization of anhydrous lactose without adsorbing moisture, enabling increased brittle fracture and smaller particle diameter. Inclusion of a crystal forming agent, such as mannitol, in the TFF formulation of TACLAC may impart more brittleness to the particles through formation of anhydrous crystals (45).

5.3.5 Formulation density

Although each formulation was produced with 0.75% w/v solids per cubic centimeter, it cannot be assumed that all powders have identical density. Lyophilized powders, particularly ones containing hygroscopic and low T_g materials, have been shown to be susceptible to collapse, shrinkage, and meltback (46). While moisture sorption is normally monitored in lyophilized products to ensure protein or chemical stability, in this study the effect of moisture sorption on powder cohesiveness and particle density are of primary interest.

Bulk and tap density testing, as defined by the USP, were used to characterize density of each powder formulation (Table 5a). While all densities measured were extremely low, the bulk density of TACLAC was approximately twice that of the other formulations, most likely due to matrix water absorption and subsequent particle contraction. Tap density was also measured and used to calculate Carr's index. Carr's index, or compressibility index, is used to describe a ductile material that undergoes plastic deformation or a brittle material that fractures under an applied force (47). Assuming that all changes in powder density were due to brittle fracture, this data

provided another indication that TACRAF is the most brittle of the powders investigated, showing a Carr's index of 50.

For comparison with USP density testing, correlation between size distribution data produced by cascade impaction (NGI) and laser diffraction (Spraytec®) analysis were also used to determine microparticle density. Knowing both the MMAD and the volumetric median diameter ($D_{[50]}$), equation 1 was used to calculate the skeletal density of the sheared microparticles exiting the DPI. Approximation of the shape factor was necessary due to its effect on aerodynamic diameter, and was assumed to be 1.5. SEM images (Figure 2) portrayed a jagged and irregular morphology of the aerosolized particles, similar that of a sand particle, which has a dynamic shape factor of 1.57 (2). Calculation of particle density proved to be slightly lower that measured by bulk density testing; however, comparing formulations to one another showed a similar relationship (Table 5b). It is possible that a lower prediction based on emitted aerosols was due to non-emitted particles remaining in the device that were excluded from characterization. Relative to each other, TACMAN and TAC produced the lowest density particles, most likely due to their non-hygroscopic nature, while TACRAF, and particularly TACLAC, showed higher density. Changes in skeletal densities of lactose, and perhaps raffinose, upon exposure to ambient moisture are due to their tendency to adsorb water and could be explained by two mechanisms. It is likely that adsorbed water effectively plasticizes the fragile matrix causing lowering of the glass transition temperature (T_g) and relaxation of supporting structures, subsequent collapsing the particle. Increased mobility of amorphous material will also lead to formation of a more thermodynamically stable, crystalline form. Powder collapse due to low material T_g has been observed previously in sucrose formulations, where inclusion of Dextran-40 significantly increased the T_g and resulted in improved structural integrity and longer stability (48). By increasing T_g of the

matrix material, molecular mobility would be limited resulting in reduction of particle shrinkage and crystal formation. Another possibility exists for particle collapse at high humidity (< 65%) where the material becomes deliquescent, partially dissolving in adsorbed moisture and effecting the integrity of the particle (49). It is doubtful, however, that deliquescent dissolving of lactose is a viable cause for particle collapse since the critical relative humidity needed for this to occur is 99% RH. Mannitol and tacrolimus, being non-hygroscopic and hydrophobic, respectively, do not experience noticeable changes in skeletal density over time due to less water adsorption and a higher T_g.

It is clear that hygroscopicity plays a large role in the final skeletal density of these low-density powders. Even if packaging and storage conditions are maintained at 0% RH, it is conceivable that highly hygroscopic particles may show substantial changes in density and geometric diameter when exposed to the humidity of the airways; although, reduction of density and geometric diameter might not have any impact of aerodynamic diameter. Due to moisture sensitivity, the density of TFF produced matrices are difficult to predict and cannot be assumed to be equivalent to the dissolved solids concentration of the TFF processed solution. Non-hygroscopic materials, such as mannitol, may allow for greater control of skeletal density through dissolved solids concentration and may allow for the creation of brittle matrices with tailored densities.

While aerosolization of brittle sacchirides, lactose and raffinose, showed superior performance, selection of appropriate formulation will depend on intended storage conditions and dosing requirements. As hygroscopic materials, both lactose and raffinose are susceptible to ambient moisture and should be packaged in completely dry conditions. Formulation with mannitol proved to be influenced less by humidity than others, however; the 100% potent TAC formulation proved to behave similarly. The only benefit to mannitol in this regard may be in stabilizing the amorphous form of the drug

for increased solubility (34). Long term stability studies are still necessary to determine any additional performance variability of these formulations.

5.4 CONCLUSION

Inhalation of low-density microparticles formed from brittle matrices with a marketed DPI device is a viable platform for highly efficient deep lung delivery of drugs. Unlike delivery strategies that utilize preformed particles, the brittle matrix TFF powders are sheared into extremely low-density (0.05 – 0.01 g/cm^3) microparticles *in situ* by patient inspiration. After incorporation of biocompatible materials such as pharmaceutical sugars into the formulations, aerosolization of the resulting brittle matrices produced fine particle fractions (FPF) as high as 70.3% and total emitted doses (TED) consistently higher than 95%.

Additional benefits of this platform for inhalation therapeutics include solubility enhancement for amorphous particles, rapid dissolution for high surface area sub-500 nm primary structures, and the ability to formulate process-sensitive actives with TFF. Future studies focusing on dose consistency, in vivo characterization, and process scale up will be investigated to determine the viability of this platform as an alternative to large porous particles and traditional carrier-based formulation.

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Tables

Table 1.1: Calcineurin inhibitor delivery methods for transplant immunosuppression

Route	Drug	Delivery technology	Improvements	Brand name	References *
Oral	Cyclosporine	drug solubilized in emmulsion	n/a	Sandimmune®	[15]
		In situ microemulsion	improved bioavail., less variability	Neoral®	[16]
		Charged nanoparitlces	improved bioavail.		[21]
		polycaprolactone nanoparticles	target lyphoctes, improved bioavail.		[19]
		polymeric micelles	Caco-2 permeability, enhanced solubility		[22]
		pH sensitive nanopatricles	targeted area of GI tract, improved bioavail.		[23]
		polymeric microspheres	controlled release, enhanced solubility		[25]
		stabilized amorphous particles	enhanced solubility		[27], [28]
	Tacrolimus	stabilized amorphous particles	n/a	Prograf®	[35]
		hydrophilic cyclodextrin	enhanced solubility		[44]
		biodegradable nanoparticles	targeted area of GI tract		[45]
		pH sensitive nanoparticles	targeted area of GI tract		[46]
Parenteral	Cyclosorine	drug solubilized in Cremphor® EL	n/a	Sandimmune®	
		liposomal	tissue targeting, longer half life, reduced toxicity		[54]
		polymeric micelles	reduced toxicity, prolonged release, enhanced solubility		[56], [57]
		biodegradable microspheres	sustained release, reduced toxicity		[60]
	Tacrolimus	drug solubilized in emmulsion	n/a	Prograf®	
		biodegradable microspheres	prolonged release		[61]
		liposomal	tissue targeting, longer half life		[55]
Implant	Cyclosorine	polymeric reservoir	sustained release		[63]
Pulmonary	Cyclosporine	disolved in ehtanol	high lung levels, low systemic levels		[66], [67], [68]
		disolved in propylene glycol	less irritating to lung	Pulminiq™	[74], [75], [69]
		liposomal	enhanced solubility, lung retention		[77]
	Tacrolimus	metered dose inhaler	high lung levels, low systemic levels		[80], [81]
		ethanol solution	suitable for nebulization		[83]
		dispersed amorphous nanoparticles	less irritating, enhanced solubility		[87]
Sublingual	Tacrolimus	Prograf® capsule content	reduced metabolism, high systemic levels		[99], [100]

* references can be found in Section 1.8

Table 2.1: Geometric droplet size distribution of a) pure deionized water and b) tacrolimus colloidal dispersion in deionized water

a)

	Average	Standard Deviation	Minimum	Maximum
Trans (%)	50.9	5.578	36	64.1
Dv(10) (µm)	2.707	0.1368	1.964	2.906
Dv(50) (µm)	6.243	0.1673	5.875	6.677
Dv(90) (µm)	12.65	0.5501	11.54	14.86
D[3][2] (µm)	3.399	0.4364	2.291	4.588
D[4][3] (µm)	7.059	0.2233	6.625	7.805
Cv (PPM)	9.765	1.533	6.437	14.63
SSA (m^2/cc)	1.765	0.24425	1.308	2.619
Obs (%)	49.1	5.578	35.9	64

b)

	Average	Standard Deviation	Minimum	Maximum
Trans (%)	49.1	5.595	30.9	61
Dv(10) (µm)	2.825	0.2274	1.741	3.063
Dv(50) (µm)	6.55	0.1347	5.995	6.792
Dv(90) (µm)	13.33	0.4966	11.82	14.89
D[3][2] (µm)	3.576	0.5369	2.042	4.728
D[4][3] (µm)	7.059	0.1763	6.653	7.787
Cv (PPM)	10.79	1.519	7.609	15.76
SSA (m^2/cc)	1.678	0.3135	1.269	2.938
Obs (%)	50.9	5.595	39	69.1

Table 3.1: Degradation and peak retention of TAC subjected to acid, alkaline, oxidative, and thermal forced degradation

Condition	Time (hr)	Tacrolimus (%)	Retention times of degradation products (min)
Acid	1	76.4	4.3, 5.9, 6.8, 10.2
Base	1	3.3	6.1, 9.3, 12, 14.2, 19.5, 22.7
H_2O_2	1	99.4	5.4
Heat	6	97.9	3.6, 5.6, 6.6, 9.5

Table 3.2: TAC powder for redispersion a) purity and b) potency after 3 months storage

a)

	Purity (%)	Degradants (%)	Stdev
initial	99.9208	0.0792	0.0082
1 month	99.8455	0.1545	0.0003
2 month	99.8517	0.1483	0.0314
3 month	99.8268	0.1732	0.0253

b)

	Potency (%)	Stdev.
initial	48.92	1.51
1 month	48.16	0.24
2 month	46.86	0.34
3 month	47.99	0.45

Table 3.3: CBC and serum chemistry of SD rats receiving TAC colloidal dispersion for 28 days and sacrificed 24 hours (4 weeks) or 168 hours (4 weeks +1) after the final dose.

Hematologic Parameters

	Control	4 weeks	4 weeks + 1
WBC (10^3/μL)	5.1 ± 0.8	4.6 ± 1.1	6.1 ± 2
RBC (10^6/μL)	8.1 ± 0.5	8.5 ± 0.8	8.3 ± 0.4
HGB (g/dL)	15.1 ± 0.7	15.2 ± 1.4	15.5 ± 0.6
HCT (%)	50.6 ± 3.3	51 ± 4.8	51 ± 3.1
MCV (fL)	62.6 ± 1.4	60.4 ± 1.5 *	61.7 ± 2.3
MCH (pg)	18.7 ± 0.6	18 ± 0.6	18 ± 0.6
MCHC (g/dL)	29.9 ± 1	29.7 ± 0.5	30.4 ± 0.7
Platelet (10^3/μL)	743.3 ± 123.5	699.5 ± 186.3	725.5 ± 281.2
Neutrophils (cells/μL)	564.5 ± 232.3	590.1 ± 380.9	705.2 ± 301.8
Neutrophils (%)	11.4 ± 4.5	14.5 ± 13.1	12 ± 5.4
Lymphocytes (cells/μL)	4351.9 ± 730.4	3851.9 ± 1227.3	5153 ± 1820
Lymphocytes (%)	86.1 ± 3.6	81.8 ± 12.4	83.5 ± 6
Monocytes (cells/μL)	78.8 ± 69.4	99.8 ± 58.2	158.3 ± 101.8
Monocytes (%)	1.6 ± 1.5	2.1 ± 1	2.7 ± 1.5
Eosinophils (cells/μL)	27.8 ± 40.2	50.4 ± 37	105.5 ± 55.1**
Eosinophils (%)	0.5 ± 0.8	1 ± 0.5	1.7 ± 0.5**
Basophils (cells/μL)	20.9 ± 29.4	32.9 ± 39	11.3 ± 27.8
Basophils (%)	0.4 ± 0.5	0.6 ± 0.7	0.2 ± 0.4

Blood Chemistry

	Control	4 weeks	4 weeks + 1
Cholesterol (mg/dL)	86.6 ± 9.5	73.6 ± 31.6	102.5 ± 21
Glucose (mg/dL)	488.9 ± 70.4	435.5 ± 191.2	488 ± 201.3
Calcium (mg/dL)	11.1 ± 0.4	11.9 ± 1.1	11.3 ± 0.7
Phosphorous (mg/dL)	11.5 ± 3.4	10.9 ± 3.3	11.9 ± 3.2
Chloride (mg/dL)	99.8 ± 1.8	n/a	97.7 ± 3.7
Potassium (mg/dL)	9.2 ± 3.5	n/a	9.5 ± 3.2
Sodium (mg/dL)	145.8 ± 1.4	n/a	146.7 ± 2.3
A/G Ratio	1.1 ± 0.1 †	1.2 ± 0.1 ** ††	1 ± 0.1 *
B/C Ratio	30.1 ± 4.1	63.5 ± 20.7 ** ††	26.7 ± 5.7

* significant difference from control; $p < 0.05$ † significant difference from 4 weeks + 1; $p < 0.05$

** significant difference from control; $p < 0.01$ †† significant difference from 4 weeks + 1; $p < 0.01$

Table 3.4: Serum chemistry of SD rats receiving TAC colloidal dispersion for 28 days and sacrificed 24 hours (4 weeks) or 168 hours (4 weeks +1) after the final dose.

Hepatic Function

	Control	4 weeks	4 weeks + 1
ALP (U/L)	171.8 ± 35.1	190 ± 46.4	167.2 ± 17.4
ALT (U/L)	176.9 ± 303.2	125.8 ± 137.7	111.2 ± 71.4
AST (U/L)	239.4 ± 319.1	173.4 ± 161.4	171.5 ± 151.4
Bilirubin (mg/dL)	0.075 ± 0	0.0625 ± 0.1	0.05 ± 0.1

Renal Function

	Control	4 weeks	4 weeks + 1
Albumin (g/dL)	3.2 ± 0.2	3.7 ± 0.4 ** †	3.2 ± 0.2
Protein (g/dL)	6.2 ± 0.4	14.7 ± 22.3	6.6 ± 0.5
Globulin (g/dL)	3 ± 0.2	3.2 ± 0.3	3.4 ± 0.3
BUN (mg/dL)	19.8 ± 2.3	20.6 ± 2.1	18.2 ± 2
Creatinine (mg/dL)	0.7 ± 0.1	0.4 ± 0.1 ** ††	0.7 ± 0.1

* significant difference from control; $p < 0.05$

** significant difference from control; $p < 0.01$

† significant difference from 4 weeks + 1; $p < 0.05$

†† significant difference from 4 weeks + 1; $p < 0.01$

Table 4.1: Comparison of lung inflammation induced by OVA sensitization and challenge with that of NS sensitization and challenge

Group	Animal ID	Treatment	Prophylactic dose	Inflammation score
POS cntrl	BALB/c 1F1	OVA,NS,OVA	5 mL NS	3
	BALB/c 1F2	OVA,NS,OVA	5 mL NS	3
	BALB/c 1F3	OVA,NS,OVA	5 mL NS	2.5
	BALB/c 1F4	OVA,NS,OVA	5 mL NS	2.5
	BALB/c 1F5	OVA,NS,OVA	5 mL NS	2.5
	BALB/c 1F6	OVA,NS,OVA	5 mL NS	2.5
	BALB/c 1F7	OVA,NS,OVA	5 mL NS	2.5
	BALB/c 1F8	OVA,NS,OVA	5 mL NS	3
	BALB/c 1G1	OVA,NS,OVA	5 mL NS	2.5
	BALB/c 1G2	OVA,NS,OVA	5 mL NS	2.5
	BALB/c 1G3	OVA,NS,OVA	5 mL NS	2.5
	BALB/c 1G4	OVA,NS,OVA	5 mL NS	3.5
	BALB/c 1G5	OVA,NS,OVA	5 mL NS	2
	BALB/c 1G6	OVA,NS,OVA	5 mL NS	3.5
			mean score	2.7 *
			standard deviation	0.3
NEG cntrl	BALB/c 1F18	NS,NS,NS	5 mL NS	0
	BALB/c 1F19	NS,NS,NS	5 mL NS	0
	BALB/c 1F20	NS,NS,NS	5 mL NS	0
	BALB/c 1F21	NS,NS,NS	5 mL NS	0
	BALB/c 1F22	NS,NS,NS	5 mL NS	0
			mean score	0
			standard deviation	0

OVA,NS,OVA = OVA IP inj, NS prophylaxis, OVA challenge

NS,NS,NS = NS IP inj, NS prophylaxis, NS challenge

* significant difference from NS,NS,NS ($p<0.0001$)

Table 4.2: Comparison the effect of prophylactic treatment on lung inflammation induced by OVA sensitization and challenge

Group	Animal ID	Treatment	Prophylactic dose	Inflammation score
DEX	BALB/c 1G13	OVA,DEX,OVA	0.5 mg DEX / 5 mL NS	2
	BALB/c 1G14	OVA,DEX,OVA	0.5 mg DEX / 5 mL NS	3
	BALB/c 1G15	OVA,DEX,OVA	0.5 mg DEX / 5 mL NS	2
	BALB/c 1G16	OVA,DEX,OVA	0.5 mg DEX / 5 mL NS	2
	BALB/c 1G17	OVA,DEX,OVA	0.5 mg DEX / 5 mL NS	2.5
	BALB/c 1G18	OVA,DEX,OVA	0.5 mg DEX / 5 mL NS	3.5
			mean score	2.5
			standard deviation	0.6
TAC	BALB/c 1F9	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	3
	BALB/c 1F10	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	3
	BALB/c 1F11	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	2.5
	BALB/c 1F12	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	3
	BALB/c 1F13	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	3
	BALB/c 1F14	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	3
	BALB/c 1F15	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	3.5
	BALB/c 1F16	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	3
	BALB/c 1F17	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	3
	BALB/c 1G19	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	2
	BALB/c 1G20	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	2
	BALB/c 1G21	OVA,TAC,OVA	45.3 mg TACLAC / 3 mL NS	2.5
			mean score	2.8 *
			standard deviation	0.3

OVA,DEX,OVA = OVA IP inj, DEX prophylaxis, OVA challenge

OVA,TAC,OVA = OVA IP inj, TAC prophylaxis, OVA challenge

* note - scores from G significantly different from F ($p < 0.005$)

Table 4.3: Tacrolimus levels present in mice receiving a single dose (45.3 mg) of tacrolimus dispersion for nebulization.

	Lung weight (g)	TAC (µg)	Lung conc. (µg/g)
BALB/c 1	0.188	0.499	2.656
BALB/c 2	0.173	0.452	2.618
BALB/c 3	0.165	0.595	3.601
BALB/c 4	0.178	0.716	4.036
		mean	3.228 ± 0.71

Table 4.4: CBC of blood obtained after dosing and challenge

	RBC (*10^6 / µL)	WBC (*10^6 / µL)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
Neg cntrl	9.5 ± 0.7	4.1 ± 2.4	16.4 ± 7.4	81.6 ± 7.1	1.2 ± 0.4	0.8 ± 0 .8
Pos cntrl	9.2 ± 0.8	7.4 ± 7.5	23.6 ± 11.4	72.9 ± 12.1	2.7 ± 2.7	0.9 ± 0.8
DEX	9.9 ± 0.6	3.7 ± 0.8	30.5 ± 7.2 *	67.0 ± 7.4	2.5 ± 1.2	0.0 ± 0.0
TAC	9.6 ± 0.9	6.3 ± 4.7	23.3 ± 11.6	73.4 ± 12.8	3.0 ± 1.5	0.3 ± 0.7

* significant difference ($p < 0.01$) from Neg cntrl

Table 5.1: Investigation of NGI testing parameters of low density particles produced by URF.

Formulation	Test conditions		Aerodynamic properties			
	Coating	Pre-sep	TED (%)	FPF (%)	MMAD (μm)	GSD (μm)
TACLAC	no	no	98.63	81.5	2.19	2.22
TACLAC	no	yes	98.29	51.3	1.85	1.92
TACLAC	yes	no	98.14	45.6	5.25	2.41

Table 5.2: Effect of capsule type on the aerodynamic performance of brittle-matrix particles emitted.

	TED (%)	FPF (%)	MMAD (μm)	GSD
Gelatin	95.49 ± 0.77	47.93 ± 2.31	1.84	1.97
HPMC	93.92 ± 1.37	59.03 ± 2.66	1.83	1.84

Table 5.3: Aerodynamic properties of brittle-matrix particles containing tacrolimus and a pharmaceutical sugar tested after production and exposure to 50% RH.

Formulation	Condition	TED (%)	FPF (%)	MMAD (μm)	GSD (μm)
TAC	initial	97.4	54.6	4.24	2.06
	50% RH	96.5	55.9	4.14	2.11
TACRAF	initial	96.6	63.5	3.31	2.83
	50% RH	93.7	61.5	3.33	2.57
TACMAN	initial	97.3	48.4	4.55	2.10
	50% RH	94.6	56.4	4.24	2.06
TACLAC	initial	94.8	70.3	2.81	2.40
	50% RH	93.8	60.5	3.62	2.29

Table 5.4: Comparison of formulation shearing in DPI device as determined by the volume moment mean ratio of unsheared to sheared powders.

Formulation	$d_{4,3}$ (unsheared)	$d_{4,3}$ (sheared)	Shearing ratio
TAC	542.8	89.44	6.07
TACRAF	502.4	67.53	7.44
TACMAN	529.7	90.61	5.85
TACLAC	460.7	58.27	7.91

Table 5.5: Density of formulated powders based on a) USP <616> guidelines for density testing and b) measured aerodynamic and geometric diameters

a)

Formulation	Bulk density (g/mL)	Tap density (g/mL)	Hausner ratio	Carr's index
TAC	0.0186	0.0294	1.67	40.0
TACRAF	0.0212	0.0423	2.00	50.0
TACMAN	0.0147	0.0227	1.54	35.0
TACLAC	0.0350	0.0553	1.58	36.7

b)

Formulation	Initial testing				Stored at 50% RH			
	Dx(50) (μm)	MMAD (μm)	*X*	ρ (g/mL)	Dx(50) (μm)	MMAD (μm)	*X*	ρ (g/mL)
TAC	51.45	4.58	1.5	0.0119	59.61	4.47	1.5	0.0084
TACRAF	40.71	3.5	1.5	0.0111	45.31	3.8	1.5	0.0106
TACMAN	64.78	5.15	1.5	0.0095	60.3	4.39	1.5	0.0080
TACLAC	49.1	n/a	1.5	n/a	30.22	3.12	1.5	0.0160

Table A1: Patents relevant to the development of tacrolimus for inhalation

Patents						
Patent No.	File Date	Issue Date	Assignee / Inventor	Title	Claims	Justification
6,361,760	4/22/1998	3/26/2002	Fujisawa Pharmaceutical Co.	Aerosol compositions	1. An aerosol composition comprising a tricyclic compound (I) of the following formula:	
					8. A process for a preparation of the aerosol composition as claimed in claim 1, comprising; (1) kneading said tricyclic compound (I) or a pharmaceutically acceptable salt thereof with a medium chain fatty acid triglyceride of the formula CH3(CH2)nCOOH, where n is 4-10, (2) distributing the resulting kneaded mass into dispensers, and (3) filling each dispenser with said liquefied hydrofluoroalkane under cooling or elevated pressure.	No medium-chain fatty acids are used in the preparation of our formulation. No HFA is needed since our formulation is not designed for a pMDI.
6,884,433	4/14/2003	4/26/2005	Fujisawa Pharmaceutical Co.	Sustained release formulation containing tacrolimus	1. A sustained-release formulation comprising a solid dispersion composition, wherein the solid dispersion composition comprises tacrolimus or its hydrate, in a mixture comprising a water-soluble polymer and a water-insoluble polymer, and an excipient.	Our formulation is not intended for sustained release and does not incoporate polymeric excipients.
6,780,324	3/18/2002	8/24/2004	Labopharm, Inc.	Preparation of sterile stabilized nanodispersions	1. A process for the production of a loaded micelle containing a biologically active agent comprising: forming a solution including at least one dispersing agent, at least one biologically active agent, and at least one solvent; lyophilizing said solution wherein a solid product is formed; and rehydrating said solid product; whereby said loaded micelle is produced.	Lyophilization is required to produce our formulation; however, no micelles are used to inhance solubility.
					15. A process in accordance with any one of claim 1 or 3 or 5 or 7 wherein said biologically active agent is at least one hydrophobic pharmaceutical composition selected from the group consisting of paclitaxel, doxorubicin, melphalan, docetaxel, teniposide, etoposide, daunomycin, vinblastine, indomethacin, ibuprofen, cyclosporine, tacrolimus, biphenyl dimethyl dicarboxylate, ketoconazole, amphotericin B, fenobibrate, and combinations thereof.	see claim 1
7,306,787	3/12/2002	12/11/2007	Nektar Therapeutics	Engineered particles and methods of use	1. A method of delivering a therapeutic dose of a bioactive agent to the pulmonary air passages in a single breath, the method comprising: providing a receptacle containing a mass of particles, the particles comprising a bioactive agent and having perforated microstructures with a bulk density of less than 0.5 g/cm3; and administering the particles as an aerosol from the receptacle to a subject's respiratory tract; wherein the particles are sized and shaped so that at least 50% of the mass of the particles are delivered to the subject's respiratory tract and wherein the fine particle fraction of particles of the aerosol is greater than 60% w/w.	Particles in our pulmonary formulaiton are not intended to be delivered via DPI.
					3. A method according to claim 2 wherein the particles comprise a geometric diameter of 1–30 microns.	Once disperse our particles are in the nanoparticle range.

Table A.1: Patents relevant to the development of tacrolimus for inhalation (continued)

Patents						
Patent No.	File Date	Issue Date	Assignee / Inventor	Title	Claims	Justification
5,817,333	5/22/1995	10/6/1998	Fujisawa Pharmaceutical Co.	Liposome perparation containing a tricyclic compound	1. A preparation comprising FK506 stably entrapped in a liposome, said liposome consists essentially of phosphatidyl choline and cholesterol.	No liposomal encapsulation is used in this formulation.
5,635,161	6/7/1995	6/3/1997	Abbott Laboratories	Aerosol drug formulations containing vegetable oil	1. A pharmaceutical composition for aerosol delivery comprising a medicament suitable for pulmonary delivery, a halogenated alkane propellant and a biocompatible C16+ unsaturated vegetable oil having an HLB value of less than 14, wherein the medicament is present in a concentration of from about 0.05% to about 5% by weight, the vegetable oil is present in a concentration of from 0.001% to about 10% by weight, the halogenated alkane propellant is selected from the group consisting of HCFC 123, HCFC 124, HCFC 141b, HCFC 225, HCFC 125, perfluorodimethylcylobutane, DYMEL 152a, HFC 134a and HFC 227ea and the biocompatible C16+ unsaturated vegetable oil is selected from olive oil, safflower oil and soybean oil.	Vegetable oil is not used to solubilize the active in our formulation. Tacrolimus is stabilized in the solid amorphous form.
5,260,301	12/1/1992	11/9/1993	Fujisawa Pharmaceutical Co.	Pharmaceutical solution containing FK-506	1. A pharmaceutical solution which comprises FK 506, a pharmaceutically acceptable surface active agent comprising polyoxyethylene hydrogenated castor oil, and a pharmaceutically acceptable nonaqueous solvent selected from the group consisting of ethanol, pyropylene glycol, glycerin and polyethylene glycol.	No castor oil or non aqueous solvent used in our formulation. Tacrolimus is stabilized in the solid amorphous form.
7,250,420	11/24/2003	7/31/2007	Pfizer Inc	Method of treatment of transplant rejection	23. A method according to claim 19, wherein the one or more additional agents is selected from the group consisting of cyclosporin A, rapamycin, tacrolimus, leflunomide, deoxyspergualin, mycophenolate mofetil, azathioprine, daclizumab, muromonab-CD3, antithymocyte globulin, aspirin, acetaminophen, ibuprofen, naproxen, piroxicam, prednisolone and dexamethasone.	Tacrolimus is the primary agent in our formulation.
6,440,458	3/25/1999	10/7/1999	Fujisawa Pharmaceutical Co.	Sustained released preparations	1. A sustained-release formulation comprising tacrolimus or its hydrate wherein the time (T63.2%) required for 63.2% of the maximum amount of tacrolimus or its hydrate to be dissolved is 0.7 to 15 hours, as measured according to the Japanese Pharmacopoeia, the 13-th edition, Dissolution Test, No. 2 (Puddle method, 50 rpm) using a test solution which is an aqueous 0.005% hydroxypropyl cellulose solution adjusted to pH 4.5, which comprises a solid dispersion composition, wherein tacrolimus or its hydrate is present as an amorphous state in water-insoluble polymer.	Peak tacrolimus concentrations are reached at approximately 30 min in our formulation; it is not sustained release The formulation is composed of amorphous tacrolimus in a water solube disaccharide.
					4. The sustained-release formulation in claim 1, wherein the solid dispersion composition is characterized by (1) lactose or calcium hydrogen phosphate is contained as an excipient and/or lubricant, and (2) the particle size of the said solid dispersion composition is equal to or smaller than 350 μm.	Our formulation is a solid solution of tacrolimus and lactose.

Table A.1: Patents relevant to the development of tacrolimus for inhalation (continued)

Patents						
Patent No.	File Date	Issue Date	Assignee / Inventor	Title	Claims	Justification
					5. The sustained-release formulation in claim 4, wherein the solid dispersion composition is characterized by (1) tacrolimus or its hydrate is present as an amorphous state in ethylcellulose, (2) lactose is contained as an excipient, and (3) the particle size of the said solid dispersion composition is equal to or smaller than 250 μm.	Lactose is used in our formulation to stabilize amorphous tacrolimus.
6,576,259	10/17/2001	6/10/2003	Fujisawa Pharmaceutical Co.	Sustained release formulation containing tacrolimus	1. A sustained-release formulation comprising tacrolimus or its hydrate, wherein the time (T63.2%) required for 63.2% of the maximum amount of tacrolimus or its hydrate to be dissolved is 0.7 to 15 hours, as measured according to the Japanese Pharmacopoeia, the 13-th edition, Dissolution Test, No. 2 (Puddle method, 50 rpm) using a test solution which is an aqueous 0.005% hydroxypropyl cellulose solution adjusted to pH 4.5.	Peak tacrolimus concentrations are reached at approximately 30 min in our formulation; it is not sustained release
					2. The sustained-release formulation in claim 1, which comprises a solid dispersion composition, wherein the tacrolimus or its hydrate is present as an amorphous state in a solid base.	Our formulation is a solid solution of tacrolimus and lactose.
					4. The sustained-release formulation in claim 2, in which the solid base is selected from a group consisting if water-soluble polymer and wax.	No wax or polymeric material is used.

Table A.2: Patent applications relevant to the development of tacrolimus for inhalation

Patent Applications						
Patent App. No.	File Date	Pub. Date	Assignee / Inventor	Title	Claims	Justification
2006/0159766	15-Dec-05	20-Jul-06	Elan	Nanoparticulate tacrolimus formulations	1. A nanoparticulate tacrolimus formulation comprising: (a) particles of tacrolimus having an effective average particle size of less than about 2000 nm; and (b) at least one surface stabilizer.	No surface stabilizer (as defined in claim 13) is used in our formulation.
					13. The composition of claim 1, wherein the surface stabilizer is selected from the group consisting of an anionic surface stabilizer, a cationic surface stabilizer, a zwitterionic surface stabilizer, a non-ionic surface stabilizer, and an ionic surface stabilizer	Lactose is not considered a surface stabilizer as described in this claim.
					30. A method of making a tacrolimus composition comprising contacting particles of tacrolimus with at least one surface stabilizer for a time and under conditions sufficient to provide a tacrolimus composition having an effective average particle size of less than about 2000 nm.	see claim 31
					31. The method of claim 30, wherein the contacting comprises grinding, wet grinding, homogenizing, or precipitation.	Rapid freezing technology is used to make our formulation; none of the processes listed in claim 31 are used.
2006/0210638	16-Mar-06	9/21/2006	Elan	Injectable compositions of nanoparticulate immunosuppressive compounds	1. An injectable nanoparticulate formulation comprising: (a) particles of tacrolimus having an effective average particle size of less than about 2000 nm; and (b) at least one surface stabilizer.	No surface stabilizer is used in our formulation. Our formulation is not intended for injection.
2006/0154953	1/5/2006	7/13/2006	Keri et al.	Amorphous tacrolimus and preparations thereof	1. Amorphous tacrolimus in a free drug particulate form.	Amorphous tacrolimus is manufactured with lactose, and is not a free drug particle in our formulation.
					7. A process for preparing amorphous tacrolimus, comprising dissolving tacrolimus in an organic polar solvent, and removing the organic polar solvent to obtain amorphous tacrolimus.	Organic polar solvent is used, however; stabilizing lyoprotectant is used to produce a stable, amorphous product
					12. The process of claim 7, wherein the solvent is removed by evaporation.	Evaporation is not used for solvent removal; sublimation is used.
2006/0177500	7/9/2004	8/10/2006	Shin et al.	Solid dispersion of tacrolimus	1. A solid dispersion comprising tacrolimus and solid surfactant having a property of hydrophile lipophile balance (HLB) value higher than or equal to about 7.	No surfactant used in our formulation. Latose is not a surfactant.
2006/0287352	8/30/2004	12/21/2006	Holm et al.	Modified release compositions comprising tacrolimus	1. Solid composition comprising an active ingredient selected among tacrolimus and analogues thereof, wherein less than 20% w/w of the active ingredient is released within 0.5 hours, when subjected to an in vitro dissolution test using USP Paddle method and using	Our formluation is not intended for oral delivery and is not a modified release formulation.

Table A.2: Patent applications relevant to the development of tacrolimus for inhalation (continued)

Patent Applications						
Patent App. No.	File Date	Pub. Date	Assignee / Inventor	Title	Claims	Justification
2006/0035918	11/4/2003	2/16/2006	Fujisawa Pharmaceutical Co.	Use of tacrolimus (fk506) derivatives combined with beta2-agonist for the treatment of asthma	2. A method for treating or preventing acute or chronic asthma, by administering an effective amount of an FK506 derivative and an effective amount of a β2-agonist to a human being or an animal.	In our formulation tacrolimus is the primary agent; there is no coadministration of additional drugs.
2002/0102294	11/12/1998	8/1/2002	Bosch et al.	Aerosols comprising nanoparticle drugs	1. An aerosol composition of an aqueous dispersion of nanoparticulate drug particles, wherein: (a) essentially each droplet of the aerosol comprises at least one nanoparticulate drug particle; (b) the droplets are of a respirable size; and (c) the nanoparticulate drug particles comprise a poorly soluble crystalline drug, have an effective average particle size of less than about 1000 nm, and have a surface modifier adsorbed on the surface thereof.	The aerosol intended in our formulatoin contains a dispersion of amorphous drug. No surface modifier is used.
					2. The aerosol composition of claim 1, wherein the drug is selected from the group consisting of proteins, peptide, bronchodilators, corticosteroids, elastase inhibitors, analgesics, anti-fungals, cystic-fibrosis therapies, asthma therapies, emphysema therapies, respiratory distress syndrome therapies, chronic bronchitis therapies, chronic obstructive pulmonary disease therapies, organ-transplant rejection therapies, therapies for tuberculosis and other infections of the lung, fungal infection therapies, respiratory illness therapies associated with acquired immune deficiency syndrome, an oncology drug, an anti-emetic, an analgesic, and a cardiovascular agent.	see claim 1
2003/0185838	5/8/2003	10/2/2003	Podolsky	Methods and compositions for treating lesions of the respiratory epithelium	16. The method of claim 12, wherein said second therapeutic agent is an anti-inflammatory agent, antimicrobial agent, antihistamine, neurokinin receptor antagonist, leukotriene receptor antagonist, decongestant, cholinergic receptor antagonist, phosphodiesterase inhibitor, or beta-adrenergic bronchodilator.	Tacrolimus is the primary and only therapeutic agent in our formulation.
2006/0124903	6/8/2004	6/15/2006	Astellas Pharma Inc.	Aerosol preparation comprising sealed container and enclosed therein aerosol composition containing marcolide compound	1. An aerosol preparation, comprising: an aerosol composition containing a macrolide compound; and an enclosure enclosing the aerosol composition, the enclosure including a valve part having a gasket comprising at least one resinous material selected from the group consisting of butyl rubber, ethylene-propylene rubber, chloroprene rubber, polyethylene, polybutylene terephthalate, polyacetal, polyamide, polytetrafluoroethylene, polypropylene and thermoplastic elastomer.	Our formulation is not a pMDI, valved, or inclosed formulation.
2005/0244339	4/14/2005	11/3/2005	Pari GmbH	Pharmaceutical aerosol composition	1. Sterile composition for administration as an aerosol, comprising a poorly water-soluble active agent, a non-ionic surfactant component and a phospholipid component, wherein the active agent is not a surfactant.	No non-ionic surfactants or phospholipids are incorporated in our formulation

Table A.2: Patent applications relevant to the development of tacrolimus for inhalation (continued)

Patent Applications						
Patent App. No.	File Date	Pub. Date	Assignee / Inventor	Title	Claims	Justification
2006/0128739	6/24/2005	6/15/2006	Maryanoff et al.	Solvent free amorphous rapamycin	1. A method of preparing amorphous rapamycin comprising: dissolving rapamycin in 2-propanol to form a solution; forming an amorphous rapamycin precipitate by adding water to the solution; and drying the amorphous rapamycin precipitate for a predetermined period of time.	Our amorphous formulation is not formed by precipitation methods; it is formed by a rapid freezing method.
					12. An amorphous therapeutic agent administerable orally, parentarally, intravascularly, intranasally, intrabronchially, transdermally, rectally or via a stent coated therewith, the amorphous therapeutic agent formed by the process comprising: dissolving an amount of crystalline therapeutic agent in a solvent to form a solution; adding an agent to the solution to precipitate the therapeutic agent from the solution; filtering the precipitate; washing the precipitate to remove impurities; and drying the precipitate.	Our amorphous formulation is not formed by precipitation methods; it is formed by a rapid freezing method.
					19. The method of claim 12, wherein the crystalline therapeutic agent is a variant of rapamycin.	see claim 20
					20. The method of claim 19, wherein the variant of rapamycin includes at least all analogs, derivatives and conjugates that bind to FKBP12, and other immunophilins.	According to this definition, tacrolimus is a variant of rapamycin.
2006/0094744	9/28/2005	5/4/2006	Maryanoff et al.	Pharmaceutical dosage forms of stable amorphous rapamycin like compounds	1. A pharmaceutical dosage form comprising substantially amorphous rapamycin like compounds and a pharmaceutically acceptable excipient.	see claim 18
					18. A process for making a pharmaceutical dosage form comprising admixing a substantially amorphous rapamycin like compound with at least one pharmaceutically acceptable excipient.	The excipient in our formulation is processed and rendered amorphous along with tacrolimus , it is not admixed.
2008/0132533	12/30/2005	6/5/2008	GL PharmTech Corp.	Solid dispersion comprising tacrolimus and enteric coated macromolecule	1. A rapidly soluble tacrolimus solid dispersion formulation comprising tacrolimus and enteric polymer, wherein the weight ratio of tacrolimus and the enteric polymer is in the range of 1:0.8˜1.2, and the latter is selected from the group consisting of hydroxypropylmethylcellulose phthalate, enteric acrylate copolymer, hydroxypropylmethylcellulose acetate succinate or carboxymethylethylcellulose.	Our formulation is rapidly soluble; however does not incorperate an enteric polymer.
2003/0235614	4/14/2003	12/25/2003	Fujisawa Pharmaceutical Co.	Sustained-release formulation	1. A sustained-release formulation of a macrolide compound, wherein the time (T63.2%) required for 63.2% of the maximum amount of macrolide compound to be dissolved is 0.7 to 15 hours, as measured according to the Japanese Pharmacopoeia, the 13-th edition, Dissolution Test, No. 2 (Puddle method, 50 rpm) using a test solution which is an aqueous 0.005% hydroxypropyl cellulose solution adjusted to pH 4.5.	Peak tacrolimus concentrations are reached at approximately 30 min in our formulation; it is not sustained release.

Table A.2: Patent applications relevant to the development of tacrolimus for inhalation (continued)

Patent Applications						
Patent App. No.	File Date	Pub. Date	Assignee / Inventor	Title	Claims	Justification
					3. The sustained-release formulation in claim 1, which comprises a solid dispersion composition, wherein the macrolide compound is present as an amorphous state in a solid base.	see claim 4
					4. A solid dispersion composition usable in the sustained-release formulation in claim 3, wherein the macrolide compound is present as an amorphous state in a solid base.	see claim 7
					7. The composition in claim 4, wherein the water-soluble base is a water-soluble polymer	The formulation is composed of amorphous tacrolimus in a water solube disaccharide.

Table A.3: Journal publications relevant to the development of tacrolimus for inhalation

Journals						
Journal	Edition	Compostion	Author	Title	Teachings	
J. Heart Lung Trans.	2005, 24(5):p. 538-543	pMDI aerosol (6361760)	Ingu et al.	Effects of inhaled FK 506 on the suppression of acute rejection after lung transplantation: use of a rat orthotropic lung transplantation model	Tacrolimus inhalation enhaced lung allograft surviva in rats. Graft survival at 10 puffs/day was comparable with 0.1 mg/kg/day IM injection. Lower systemic levels were seen with inhalation therapy.	Our formulaiton does not use a chemical solubility enhancer such as meduim chain triglycerides and is not designed for pMDI delivery.
J. Thoracic Cardiovasc. Surg.	2007, 133(2): p. 548-553	pMDI aerosol (6361760)	Ide et al.	Efficacy and saftey of inhaled tacrolimus in rat lung transplantation	Minimal tacrolimus concentration to prevent lung allograft rejection after inhalation is 270.4 ng/g. Associated whole blood levels were low (4.87 ng/ml). Higher blood and lung levels were achieved with IM injection.	Our formulaiton does not use a chemical solubility enhancer such as meduim chain triglycerides and is not designed for pMDI delivery.
Biochemistry	2004, 43(30): p. 9926-9938	liposomal for intratraceal	Canadas et al.	Characterization of liposomal tacrolimus in lung surfactant-like phospholipids and evaluation of its immunosuppressive activity	Tacrolimus can be incorperated to into DPPC and POPG mono and billayers up to 0.4 mol %. IL-2 production was reduced 80% by liposomal tacrolimus in culture. This is a suggested for intratracheal administation.	No lipids are used in our formulation. Solubility is enhanced throught the stabilization of amorphous drug particles.
Int. Arch Allergy Immunol	2005, 136: p. 372-378	pMDI aerosol (6361760)	Morishita et al.	FK506 aerosol locally inhibits anitgen-induced airway inflammation in guinea pigs	Inflamation inhibition was similar with 3 puffs tacrolimus pMDI and 1 mg/kg oral dose in sensitized guinea pigs. Inhalatin induced systemic levels were 1/21 that of oral systemic levels.	Our formulaiton does not use a chemical solubility enhancer such as meduim chain triglycerides and is not designed for pMDI delivery.
Am. J. Transplant.	2007, 7: p. 1733-1742	70% ethanol solution for neb	Schrepfer et al.	Effect of inhaled tacrolimus on cellular and humoral rejection to prevent posttransplant obliterative airway disease	4 mg/kg was given orally and via aerosol in tracheal transplanted rats. Inhaled tacrolimus showed blood levels 5.5 fold lower than oral formulation.	No organic solvents are used to dose our formulaiton. Solublity is enhanced through the dosing of amorphous drug particles.
J. Thoracic Cardiovasc. Surg.	2004, 127: p. 376-384	endotracheal comercial tacrolimus (Fujisawa)	Woolley et al.	Endotracheal calcineurin inhibition ameliorates injury in an experimental model of lung ischemia-reperfusion	Decreased alveolar neutrophil and cytokine expression was seen in endotracheal tacrolimus. It also reduced lung lung vascular permeability	It unclear whether Fujisawa's oral or pMDI tacrolimus was provided for this study. Regardless, there are no similarites to our formulation.
Anal. Biochem.	2005, 340: p. 57-65	flourescent tacrolimus	Canadas et al.	Equilibrium studies of a fluorescent tacrolimus binding to surfactant protein A	Flourescently labled tacrolimus was found to bind to surfactant protien A, which is found in alveolar fluid. The equilibrium constant was found to be 10^7 M^{-1}.	The drug used in this in vitro study is not formulated for aerosolization.

Table B.1: Comparison of TAC:LAC dispersion aerosol characteristics by air-jet and vibrating mesh nebulization.

Nebulizer	Dispersion Conc.	TED (µg)	TED (%)	FPF (%)	MMAD (µm)	GSD (µm)
Air Jet	5 mg/ 3 mL	98.73	5.39	58.68	2.41	2.33
	10 mg/ 3 mL	331.29	12.99	62.51	1.91	2.33
	15 mg/ 3 mL	570.89	19.20	54.94	1.91	2.45
Vibrating Mesh	5 mg/ 3 mL	785.53	50.51	44.18	4.06	2.73
	10 mg/ 3 mL	563.00	17.42	46.32	3.79	2.85
	15 mg/ 3 mL	1640.01	22.64	47.03	3.95	2.63

TED = Total emitted dose
FPF = Fine particle fraction (4.7 µm or smaller)
MMAD = Mass median aerodynamic diameter
GSD = Geometirc standard deviation

Table C.1: Nebulization of aqueous media and tacrolimus dispersion for nebulization using a) deionized water and b) normal saline as dispersion media.

A)

	aqueous only	dispersion
Trans (%)	50.90 ± 5.58	49.10 ± 5.60
Dv(10) (µm)	2.71 ± 0.14	2.83 ± 0.23
Dv(50) (µm)	6.24 ± 0.17	6.55 ± 0.13
Dv(90) (µm)	12.65 ± 0.55	13.33 ± 0.50
D[3][2] (µm)	3.40 ± 0.44	3.58 ± 0.54
D[4][3] (µm)	7.06 ± 0.22	7.06 ± 0.18
Cv (PPM)	9.77 ± 1.53	10.79 ± 1.52
SSA (m^2/cc)	1.77 ± 0.24	1.68 ± 0.31
Obs (%)	49.10 ± 5.58	50.90 ± 5.60

B)

	aqueous only	dispersion
Trans (%)	35.30 ± 4.90	59.50 ± 5.05
Dv(10) (µm)	1.32 ± 0.59	2.24 ± 0.20
Dv(50) (µm)	6.07 ± 0.19	5.54 ± 0.15
Dv(90) (µm)	12.81 ± 0.43	11.46 ± 0.50
D[3][2] (µm)	2.04 ± 0.32	3.00 ± 0.48
D[4][3] (µm)	6.80 ± 0.25	6.30 ± 0.20
Cv (PPM)	13.10 ± 1.22	6.31 ± 0.90
SSA (m^2/cc)	2.94 ± 0.05	2.00 ± 0.31
Obs (%)	64.70 ± 4.90	40.50 ± 5.05

Trans = laser transmission
D[3][2] = surface area moment mean
D[4][3] = volume moment mean
Cv = measurement concentration
SSA = specific surface area
Obs = laser obscuration

Table D.1: FK binding protein (FKBP) and ATP-binding cassette (ABC) protein interactions with tacrolimus

	binding protiens	synonyms	binding effect	binding affinity
Immunophilins	FKBP-12	FKBP1A	immunosuppression	Along with FKBP12.6, are the only immunophilins to bind with TAC and inhibit calcinuerin [1]
	FKBP-12.6	FKBP1B	immunosuppression	Along with FKBP12, are the only immunophilins to bind with TAC and inhibit calcinuerin [1]
	FKBP-13	FKBP2	insufficent binding	The affininty of TAC for FKBP-13 is about 50 fold less than FKBP-12 [2]; perdominates in mast cells [2]
	FKBP-25	FKBP3	insufficent binding	The affininty of TAC for FKBP-25 is about 200 fold less than FKBP-12 [2]; shows 8 fold more affinity for SIR [2]
	FKBP-36	FKBP6	insufficent binding	No affintiy for TAC demonstrated [2]
	FKBP-38	FKBP8	insufficent binding	Unknown
	FKBP-51	FKBP5	inhibits FKBP-51 association with the GR heterocomplex [3], [4]; overexpression dose not effect TAC immunosup [5];	The affninty of TAC for FKBP-51 is less than FKBP-12 [5]
	FKBP-52	FKBP4, FKBP59, HSP56, P59	inhibits FKBP-52 association with the androgen receptor heterocomplex [6]; TAC presence does not affect GR receptor biniding [7]	The affininty of TAC for FKBP-52 is about 100 fold less than FKBP-12 [2]; Prolyl isomerase (PPI) activity is the same as FKBP-12 [2]
ATP-binding cassette (ABC) proteins	ABCG2	breast cancer resistance protein (BCRP)	inhibits - not substrate [8]	Unknown
	ABCB1	P-glycoprotein, MDR1	inhibits - competitive inhibition [9]	Unknown

[1] Wiederrecht et al; Perspect Drug Disc Design, 1994, v2, 57-84
[2] Tai et al; Science, 1992, v256, 1315-1318
[3] Reynolds et al; J Clin Endocrin Metab, 1999, v84(2): 663-669
[4] Denny et al, Endocrin, 2005, v146(7), 3194-3201l
[5] Li et al, J Cell Biochem, 2002, v84, 460-471
[6] Periyasamy et al; Endocrinology, 2007, v148(10): 4716-4726
[7] Hutchison et al, Biochem, 1993, v32, 3953-3957
[8] Gupta et al; Cancer Chemother Pharmacol, 2006, v58: 374-83
[9] Aroeci et al; Blood, 1992, v80: 1528-36

Table E.1: Raw CBC data for normal saline dosed Sprague Dawley (SD) rats

	M1C-28	F1C-28	M2C-28	F2C-28	M3C-28	F3C-28	M4C-28	F4C-28	High	Low
Treatment	NS	NS	NS	NS	NS	NS	NS	NS		
Dose frequency (per day)	1	1	1	1	1	1	1	1		
Duration (days)	28	28	28	28	28	28	28	28		
Gender	male	female	male	female	male	female	male	female		
WBC (*10^3 cell/µL)	6.6	4.5	5	5	5.1	5.6	3.9	4.7		
RBC (*10^6 cell/µL)	8.79	7.45	8.42	7.93	8.06	7.69	8.73	7.68	10	7
HGB (g/dL)	16.1	14.4	15.7	15.3	15.4	14.2	15.2	14.5		
HCT (%)	53.3	46.6	54.7	50.5	50.4	47.7	54.5	47.2	48	36
MCV (fL)	61	63	65	64	63	62	62	61		
MCH (pg)	18.3	19.3	18.7	19.3	19.1	18.4	17.5	18.9		
MCHC (g/dL)	30.2	30.9	28.8	30.3	30.5	29.7	28	30.8		
Neutrophils (%)	10	7	11(1)	11	21	8	14	8	34	9
Lymphocytes (%)	87	91	86	86	79	89	84	87	85	68
Monocytes (%)	1	1	2	1	0	1	2	5	5	0
Eosinophils (%)	1	0	0	2	0	1	0	0	6	0
Basophils (%)	1	1	0	0	0	1	0	0	2	0
Platelet (*10^3 cell/µL)	792	745	822	975	644	743	628	597		
Neutrophils (cell/µL)	660	315	550(50)	550	1071	448	546	376		
Lymphocytes (cell/µL)	5742	4095	4300	4300	4029	4984	3276	4089		
Monocytes (cell/µL)	66	45	100	50	0	56	78	235		
Eosinophils (cell/µL)	66	0	0	100	0	56	0	0		
Basophils (cell/µL)	66	45	0	0	0	56	0	0		
Polychromasia	slight	n/a	slight	n/a	slight	n/a	slight	n/a		
Anisocytosis	n/a	n/a	slight	n/a	slight	n/a	slight	n/a		
Remarks	clumped	n/a	clumped	n/a	n/a	n/a	n/a	n/a		

Table E.2: Raw serum chemistry data for normal saline dosed Sprague Dawley (SD) rats

	M1C-28	F1C-28	M2C-28	F2C-28	M3C-28	F3C-28	M4C-28	F4C-28	High	Low
Treatment	NS	NS	NS	NS	NS	NS	NS	NS		
Dose frequency (per day)	1	1	1	1	1	1	1	1		
Duration (days)	28	28	28	28	28	28	28	28		
Gender	male	female	male	female	male	female	male	female		
Alkaline Phosphatase (U/L)	150	139	181	150	232	167	215	140		
Alanine Amino Transferase (U/L)	59	47	917	191	66	36	50	49	40	30
Aspartate Amino Transferase (U/L)	67	84	719	791	81	39	65	69	75	45
Albumin (g/dL)	3	3.2	3.2	3.7	3	3	3	3.1	4.8	3.8
Total Protein (g/dL)	6	6.3	6.2	7	6.1	6.1	5.8	6	7.6	5.6
Globulin (g/dL)	3	3.1	3	3.3	3.1	3.1	2.8	2.9	3	1.8
Total Bilirubin (mg/dL)	0.1	0	0	0.1	0.1	0.1	0.1	0.1	0.5	0.2
Direct Bilirubin (mg/dL)	0.1	0	0	0.1	0.1	0.1	0.1	0.1		
Blood Urea Nitrogen (mg/dL)	18	20	16	24	20	20	19	21	21	15
Creatinine (mg/dL)	0.7	0.7	0.7	0.8	0.6	0.6	0.6	0.6	0.8	0.2
Cholesterol (mg/dL)	88	103	81	91	75	93	75	87	130	40
Glucose (mg/dL)	556	408	481	535	477	366	553	535	135	50
Ca 2+ (mg/dL)	11.3	10.7	11.6	10.9	11.5	10.6	10.9	11.2	13	5
Phosphorus (mg/dL)	10.7	8.5	17.5	14.5	11.2	7.3	13.1	9.2	8.3	5.3
Cl - (meq/L)	101	101	99	97	101	102	99	98		
K + (meq/L)	8.8	6.4	14.4	14.7	7.9	5.2	9	7.1	5.8	3.7
Na + (meq/L)	146	147	145	143	147	147	146	145	140	127
A/G Ratio	1	1	1.1	1.1	1	1	1.1	1.1		
B/C Ratio	25.7	28.6	22.9	30	33.3	33.3	31.7	35		
Indirect Bilirubin (mg/dL)	0	0	0	0	0	0	0	0		
NA/K Ratio	17	23	10	10	19	28	16	20		
Hemolysis Index	none	none	+	+	none	none	none	+		
Lipemia Index	none	none	none	none	none	none	none	none		

+ = slight hemolysis

Table F.1: Raw inflammation scores of asthma model lung tissue when treated with normal saline and tacrolimus dispersion for nebulization.

Animal ID	Treatment	Dose	Inflammation score
BALB/c 162	NS	5 mL	2.5
BALB/c 163	NS	5 mL	2.5
BALB/c 164	NS	5 mL	2.5
BALB/c 165	NS	5 mL	2.5
BALB/c 166	NS	5 mL	2
BALB/c 167	NS	5 mL	2
BALB/c 168	NS	5 mL	2
BALB/c 169	NS	5 mL	2
		mean score	2.25
		standard deviation	0.25
BALB/c 178	TAC	45.3 mg / 3 mL	1
BALB/c 179	TAC	45.3 mg / 3 mL	2
BALB/c 180	TAC	45.3 mg / 3 mL	2
BALB/c 181	TAC	45.3 mg / 3 mL	2
BALB/c 182	TAC	45.3 mg / 3 mL	2
BALB/c 183	TAC	45.3 mg / 3 mL	1.5
BALB/c 184	TAC	45.3 mg / 3 mL	2
BALB/c 185	TAC	45.3 mg / 3 mL	2
		mean score	1.81*
		standard deviation	0.34

NS = normal saline

TAC = tacrolimus

* significant difference from saline ($p<0.05$)

Table H.1: Pathological evaluation of inflammation in asthma induced mice dosed with prophylactic combination of tacrolimus and dexamethasone.

Group	Animal ID	Treatment	Prophylactic dose	Inflammation score
TAC/DEX	BALB/c 1G7	OVA,TAC/DEX, OVA	TAC + DEX	2.5
	BALB/c 1G8	OVA,TAC/DEX, OVA	TAC + DEX	2.5
	BALB/c 1G9	OVA,TAC/DEX, OVA	TAC + DEX	2
	BALB/c 1G10	OVA,TAC/DEX, OVA	TAC + DEX	2.5
	BALB/c 1G11	OVA,TAC/DEX, OVA	TAC + DEX	2
	BALB/c 1G12	OVA,TAC/DEX, OVA	TAC + DEX	2.5
			mean score	2.3 † *
			standard deviation	0.3

OVA,TAC/DEX,OVA = OVA IP inj, TAC prophylaxis, OVA challenge

† significant difference from OVA,NS,OVA ($p<0.05$)

* significant difference from OVA,TAC,OVA ($p<0.05$)

Figures

Figure 1.1: Cycle of CD4 T cell activation with sites of immunosuppressive action. (Reprinted with permission from Mueller et al, 2004).

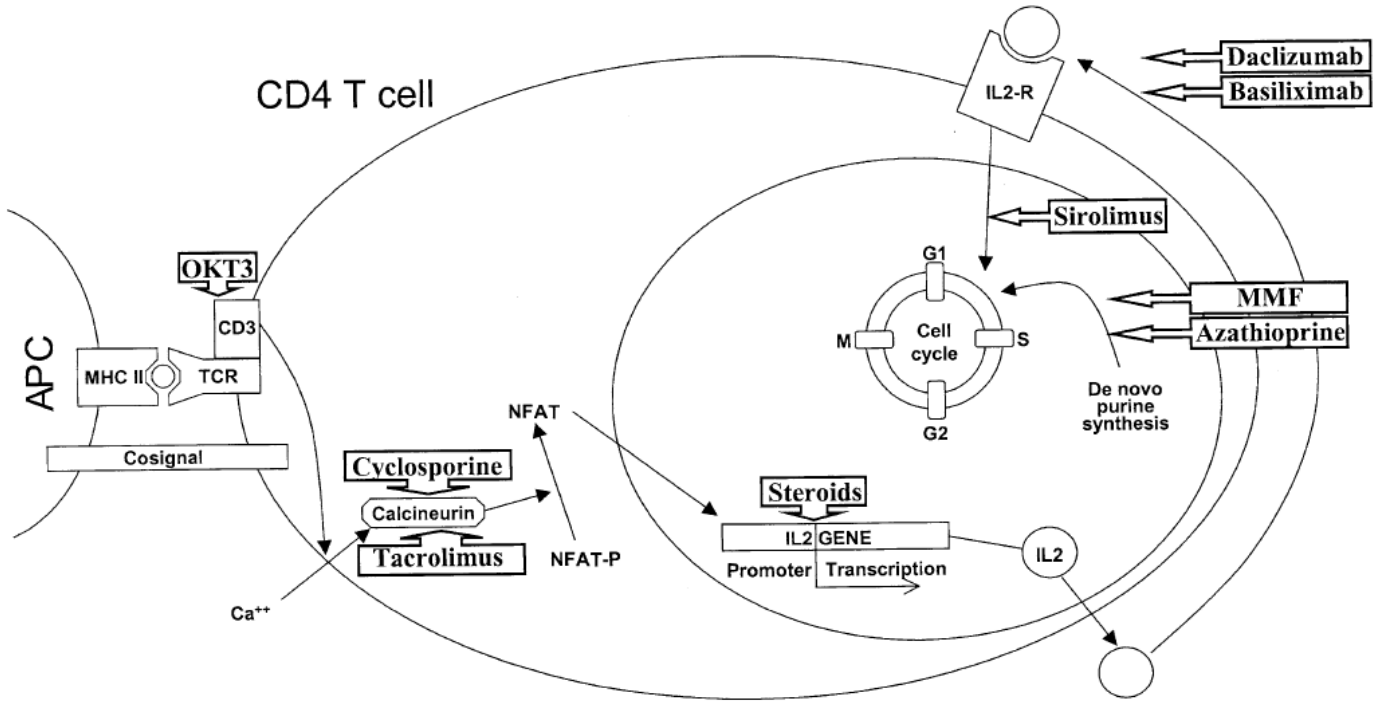


Figure 1.2: Maintenance immunosuppressive drug use in adult lung recipients: CyA, cyclosporine; TAC, tacrolimus; MMF, mycophenolate mofetil; AZA, azathioprine (Reprinted with permission from Trulock et al, 2007)

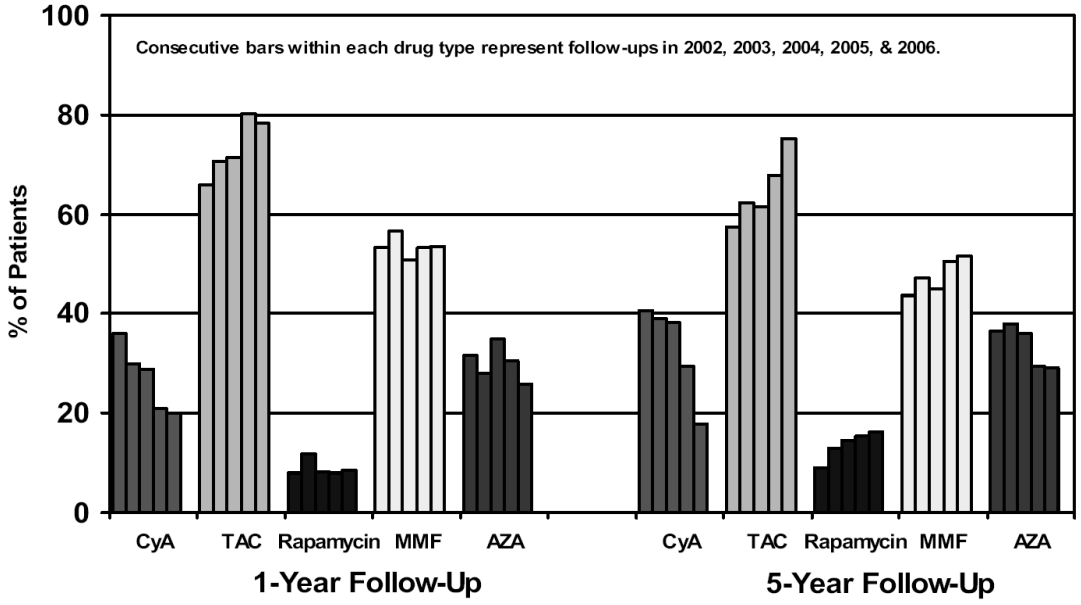


Figure 1.3: Induction therapy by year in adult lung recipients: ALG, anti-lymphocyte globulin; ATG, anti-thymocye globulin; IL-2R, interleukin-2 receptor (Reprinted with permission from Trulock et al, 2007).

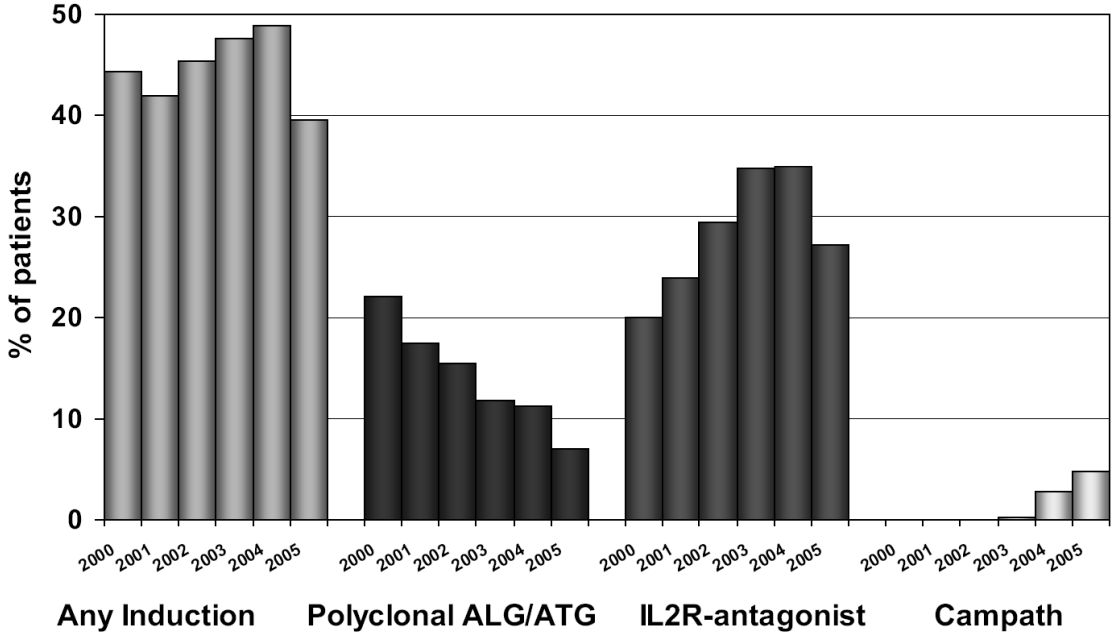


Figure 1.4: Survival of lung transplant recipients receiving aerosolized cyclosporine in addition to normal immunosuppressive maintenance therapy (Reprinted with permission from Iacono et al, 2006).

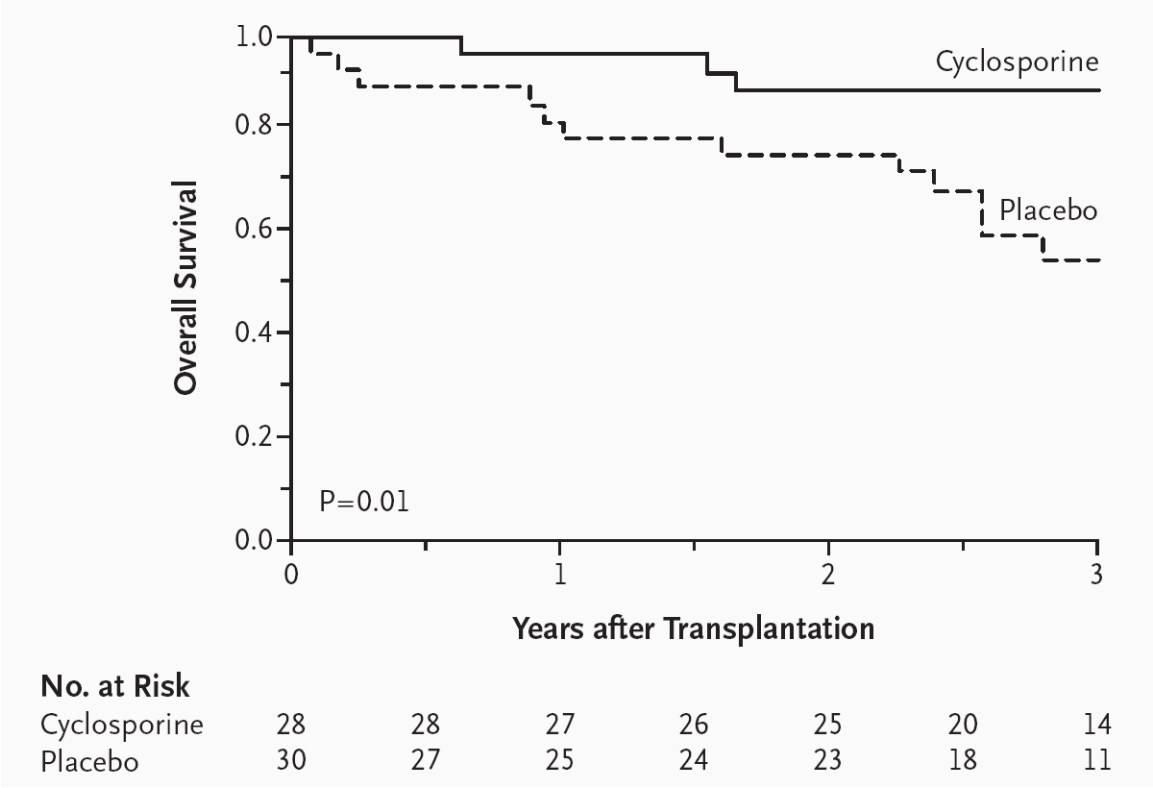


Figure 1.5: Supersaturated dissolution profile for (●) amorphous URF composition TAC:lactose (1:1); (■) crystalline URF composition TAC alone and (---) equilibrium solubility of TAC in the dissolution media fluids (SLF) containing 0.02% DPPC at 100 rpm and 37°C (Reprinted with permission from Sinswat et al, 2008).

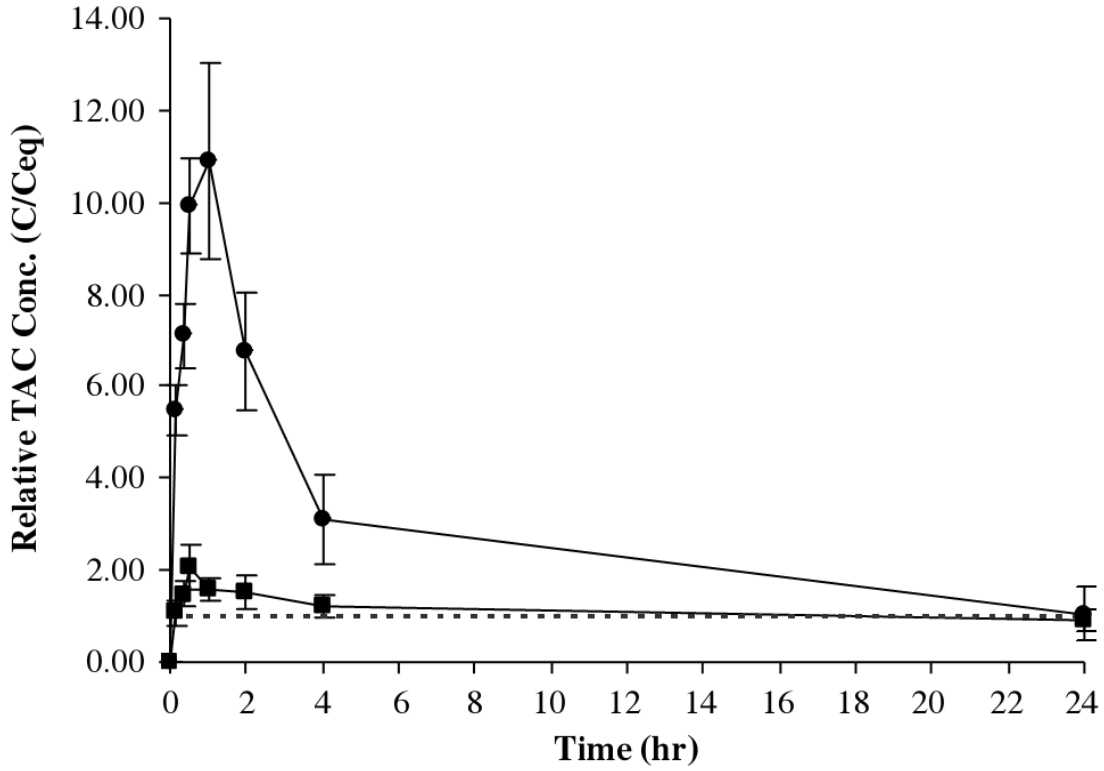


Figure 2.1: Pathologically reviewed slides of transplanted lung tissue representing a) no rejection and b) acute graft rejection at 3 days post-op and and c) no rejection and d) acute graft rejection at 6 days post-op.

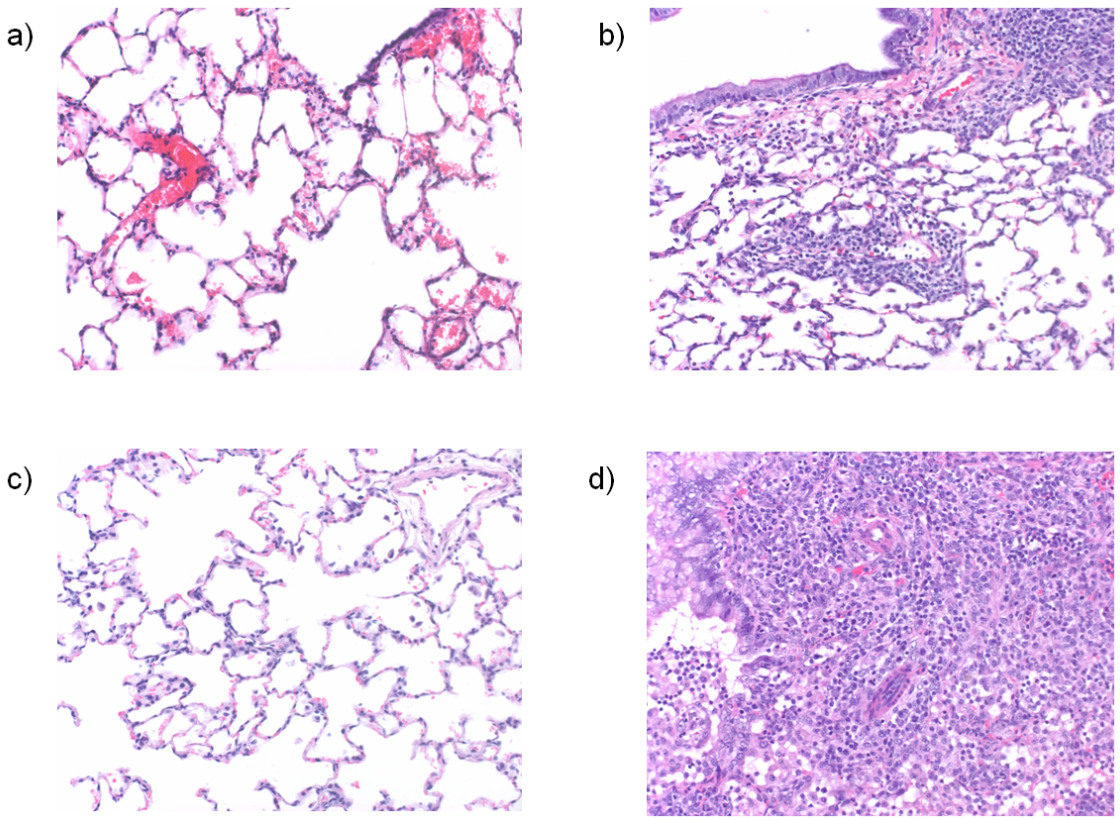


Figure 2.2: Analysis of resulting particle size of particulate drug formulation within an aqueous media (▲) 5, (■) 15, and (●) 30 minutes after dispersion via probe sonication. Particle diameter is plotted versus normalized volume distribution.

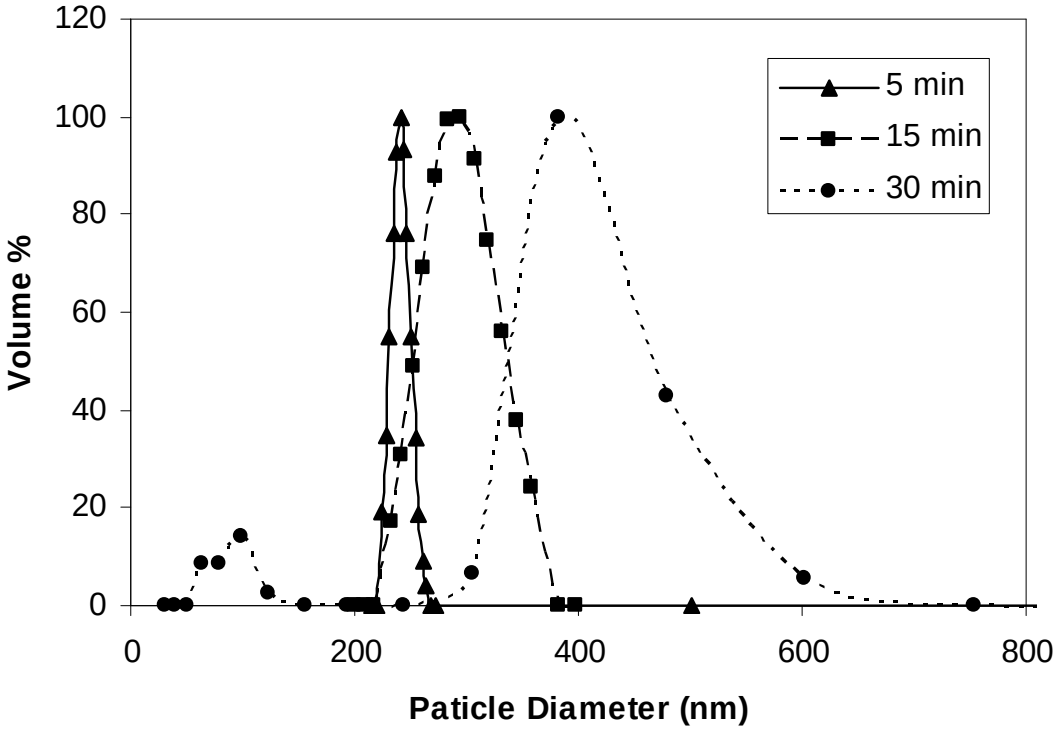
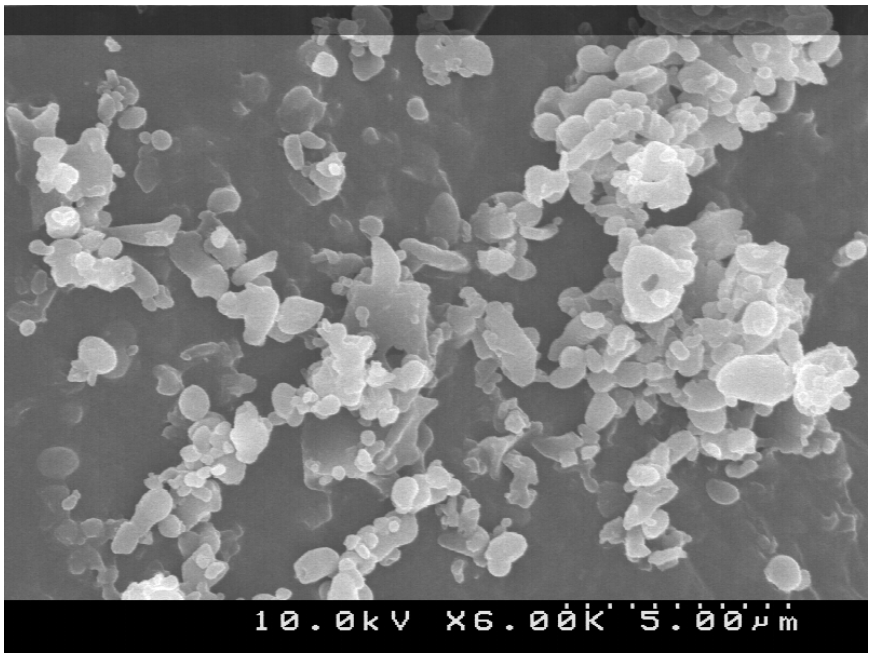


Figure 2.3: Scanning electron microscopy images at a) 6K and b) 40K magnification of tacrolimus colloidal dispersion after freezing and solvent removal.

a)



b)

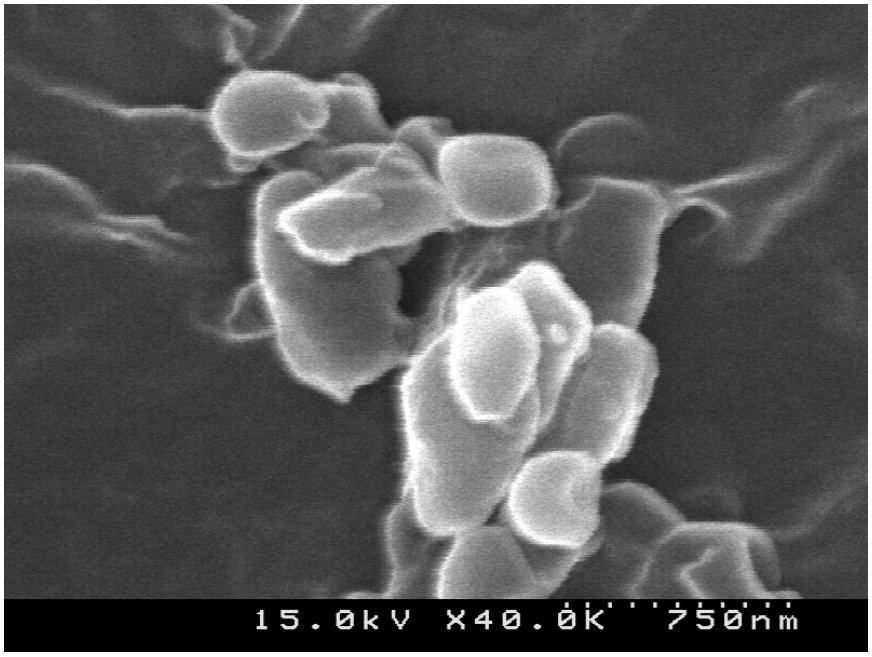


Figure 2.4: Aerodynamic droplet size of nebulized tacrolimus colloidal dispersion determined by Next Generation Pharmaceutical Impaction.

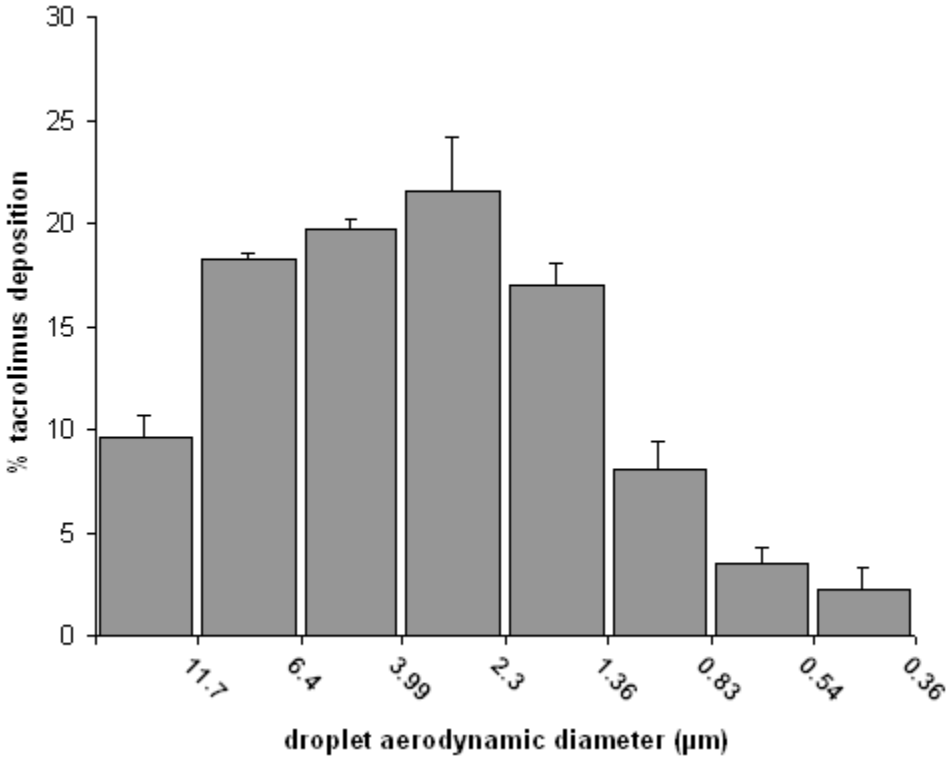


Figure 2.5: Total lung and whole blood drug concentration in transplant and non-transplanted (control) Lewis rats.

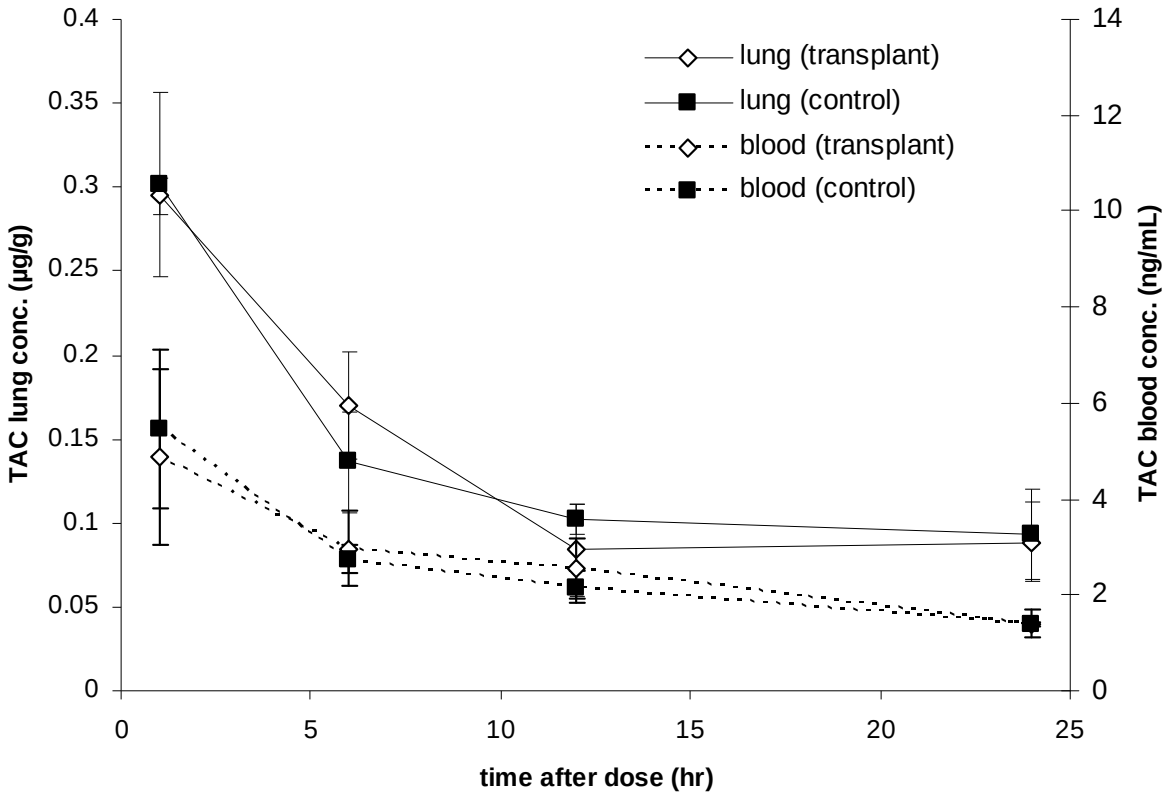


Figure 2.6: Drug concentration in the trachea of transplanted and non-transplanted (control) Lewis rats after a single dose of tacrolimus colloidal dispersion.

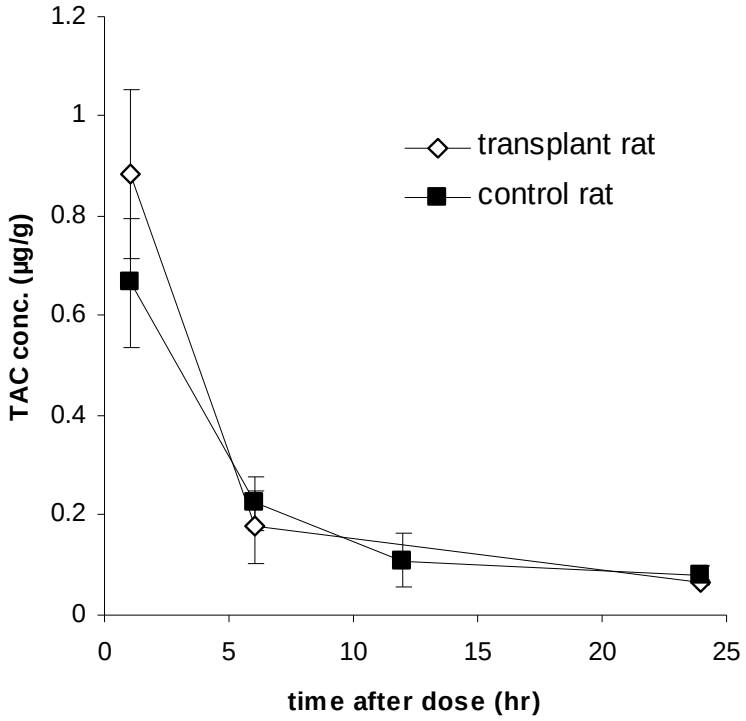
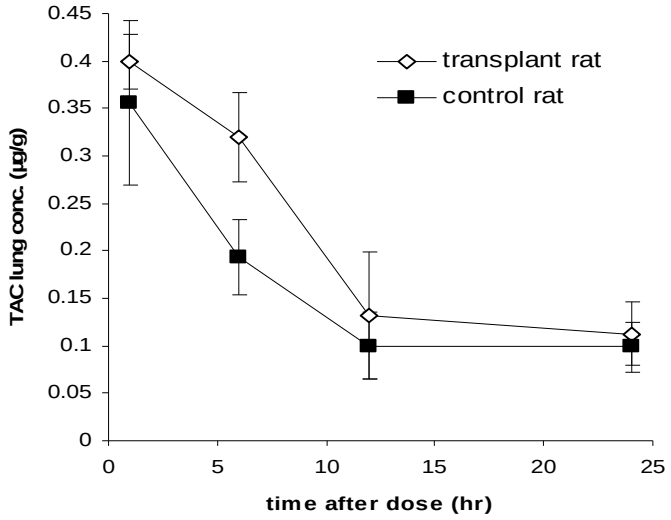


Figure 2.7: Drug concentrations in a) left and b) right lungs of transplanted and non-transplanted (control) Lewis rats after a single dose of tacrolimus colloidal dispersion. Note: Only left lungs were transplanted in transplant rats.

a)



b)

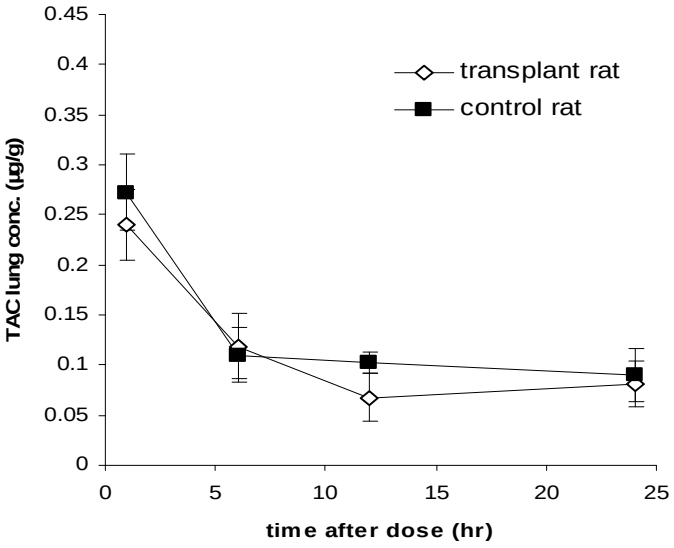


Figure 3.1: Supersaturation dissolution profile for TAC colloidal dispersion initially (△) and after 1 month (▲), 2 months (□), and 3 months (■) in simulated lung fluid (SLF) containing 0.02% DPPC at 37°C and a paddle speed of 100 rpm. Equilibrium solubility (6.8µg/mL) is represented by the dashed line (---).

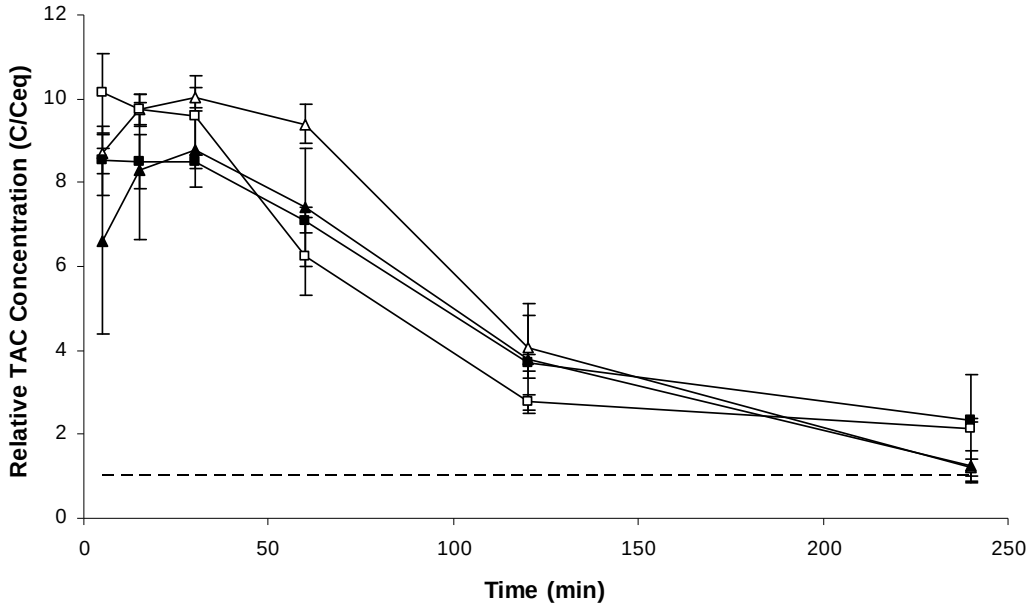


Figure 3.2: X-ray diffraction patterns of tacrolimus powder for dispersion over 3 months stability.

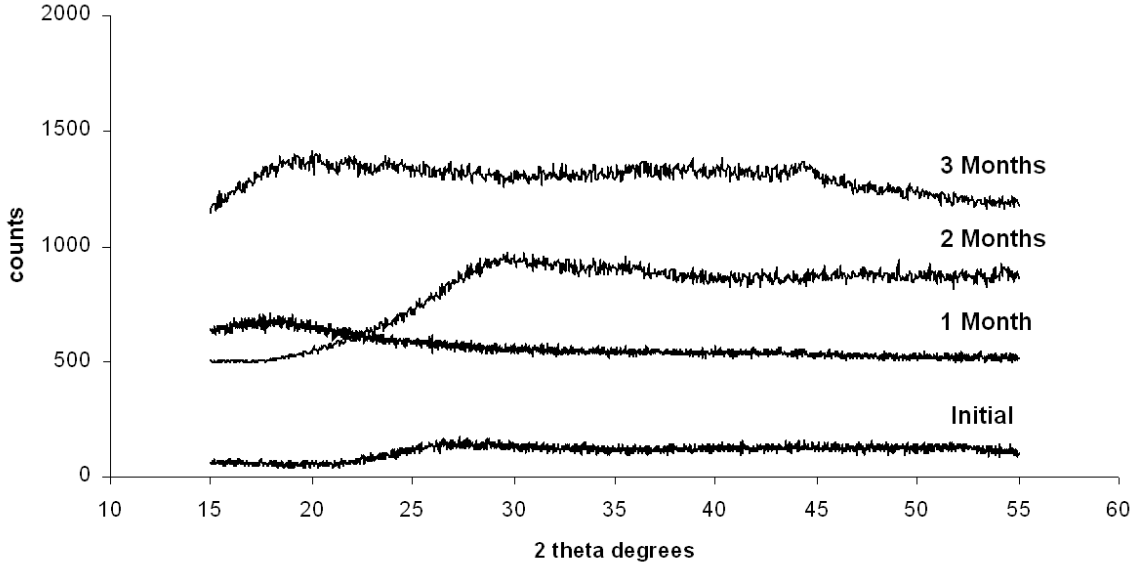
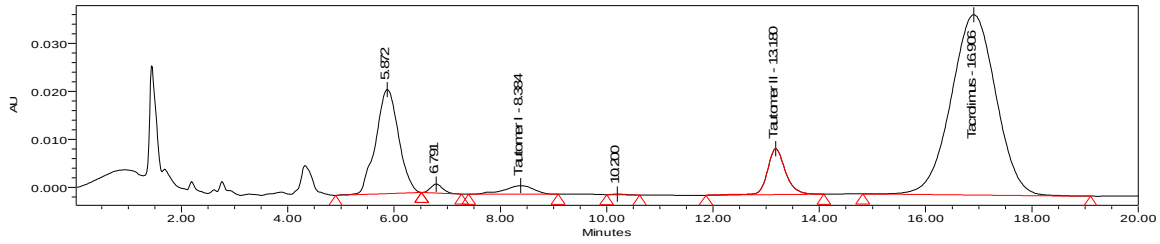
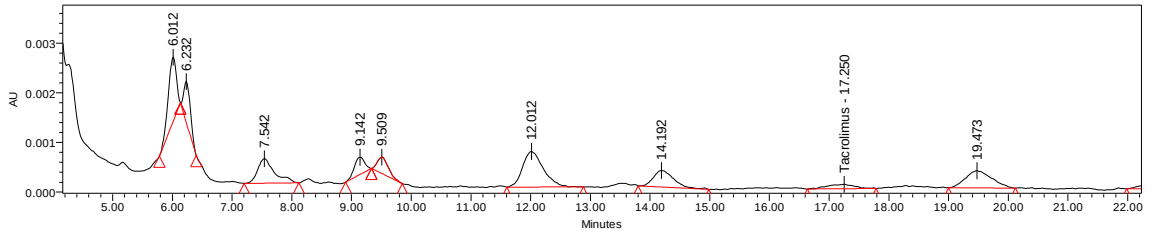


Figure 3.3. Chromatographic results from a) acidic, b) alkaline, c) oxidative, and d) thermal degradation of TAC.

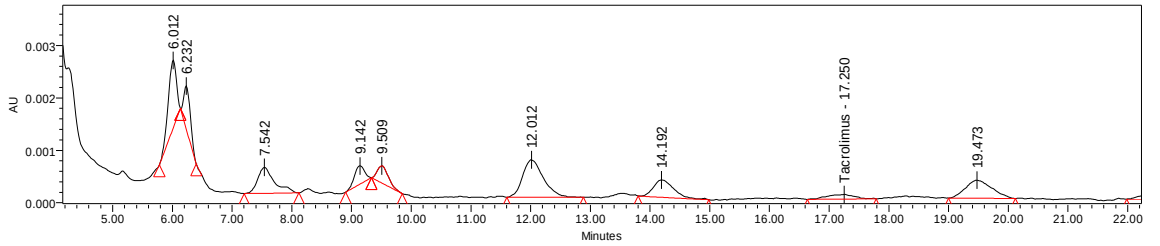
a)



b)



c)



d)

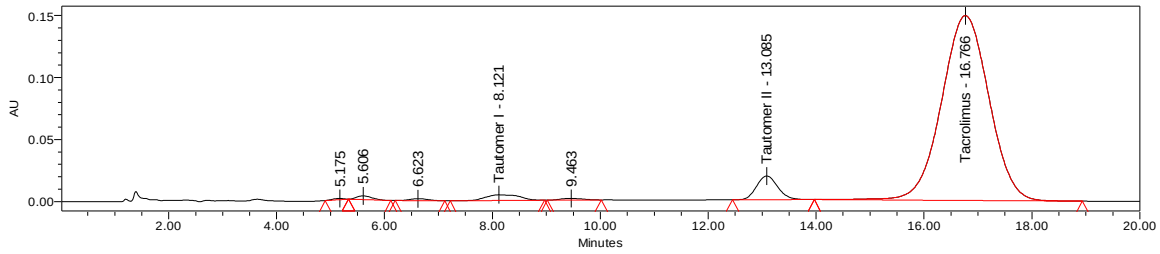


Figure 3.4: Gas chromatography of tacrolimus powder for dispersion dissolved in 1 mL DMSO

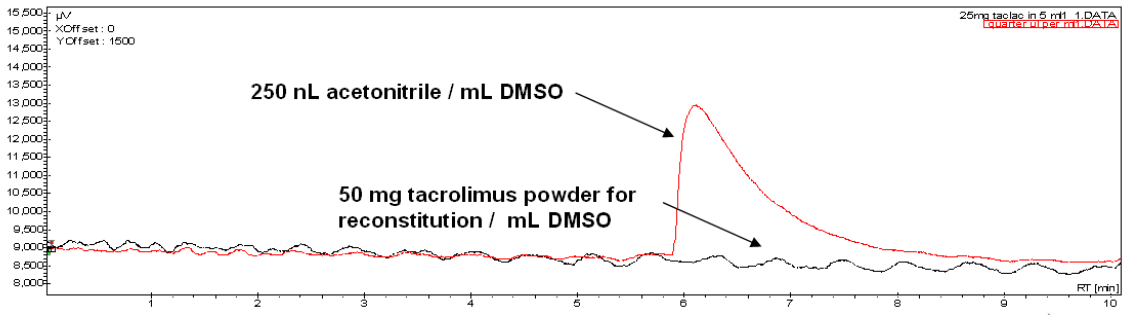
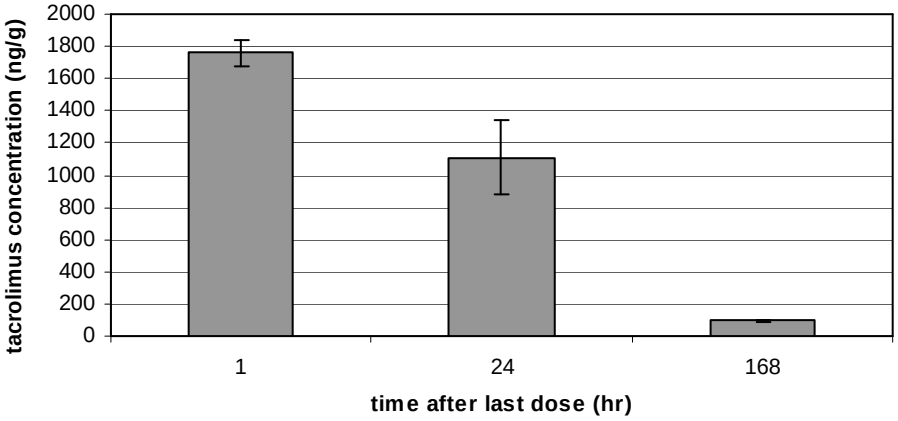


Figure 3.5: Tacrolimus concentration remaining in a) lung tissue and b) whole blood after 28 daily nebulized doses of 6.4 mg tacrolimus dispersion for nebulization.

a)



b)

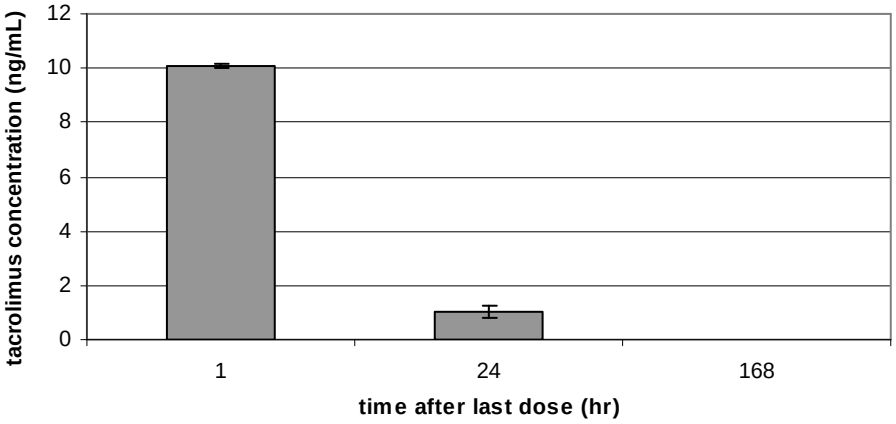
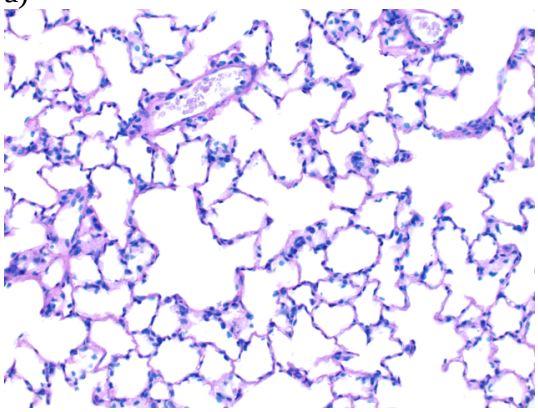
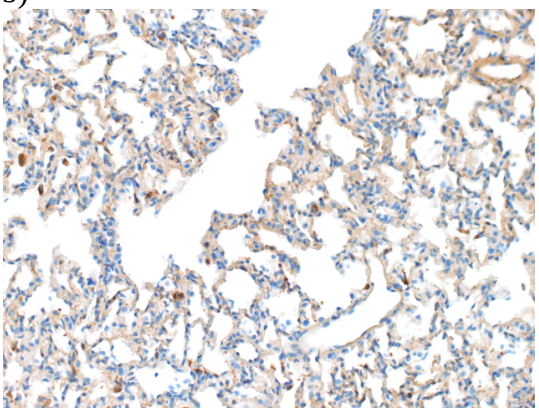


Figure 3.6: Histological staining of lungs with a) H&E, b) CD 68, and c) PAS for evaluation of cellular inflammation, monocyte/marcrophage presence, and mucus production, respectively.

a)



b)



c)

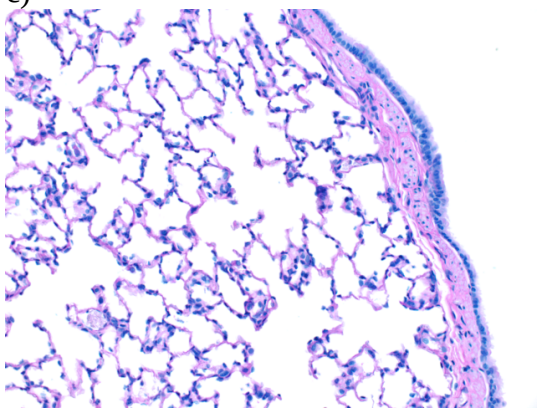


Figure 4.1: White blood cell differential count of BALF

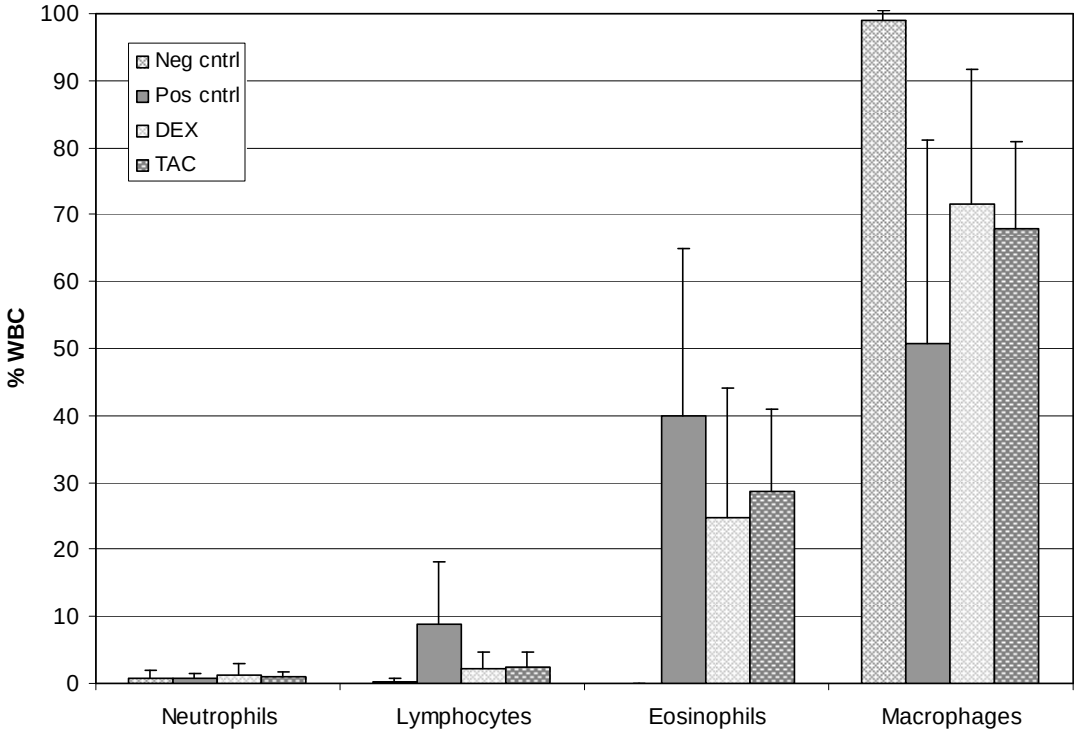
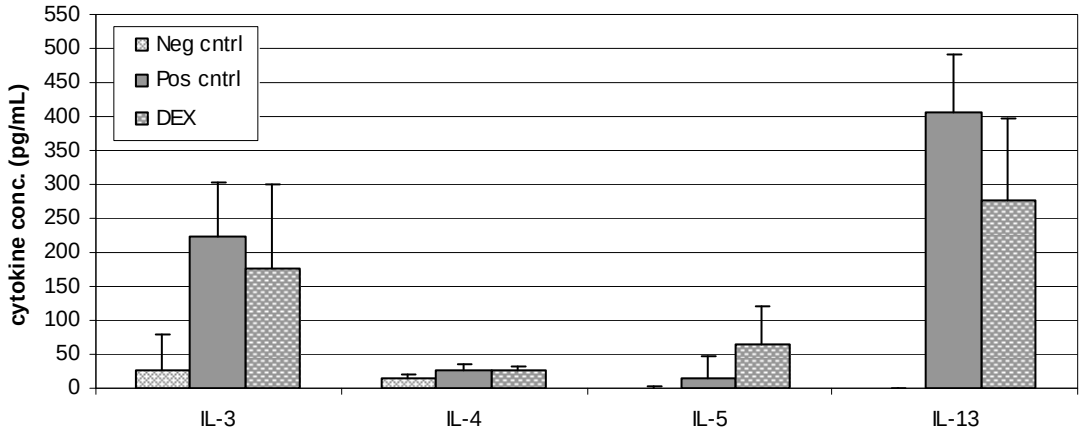


Figure 4.2: Cytokine evaluation from groups of asthma induced mice treated with a) inhaled dexamethasone solution or b) tacrolimus dispersion for nebulization

a)



b)

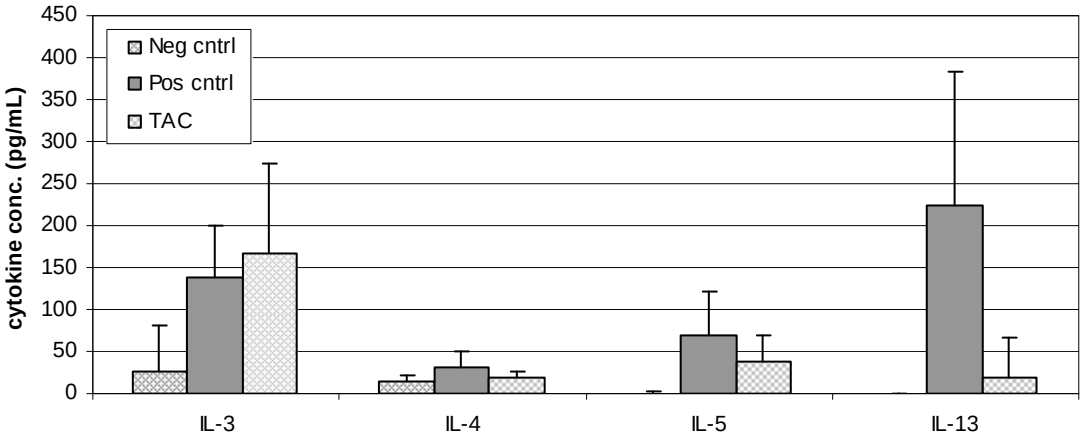


Figure 5.1: Deposition of brittle-matrix powder in a NGI tested with (light) and without (dark) a pre-separator. Percent deposition is based only on stages 2 through 7 and does not include particles collect on the pre-separator, stage 1, or MOC.

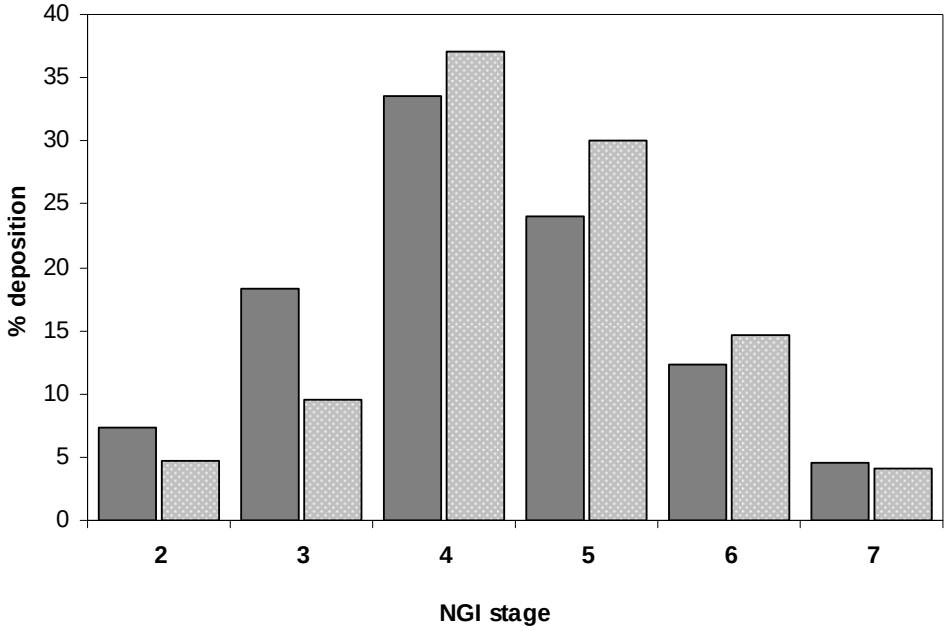


Figure 5.2: SEM images of TACLAC a) respirable brittle-matrix particles, b), c) drug/excipient network, and d) primary particle structure.

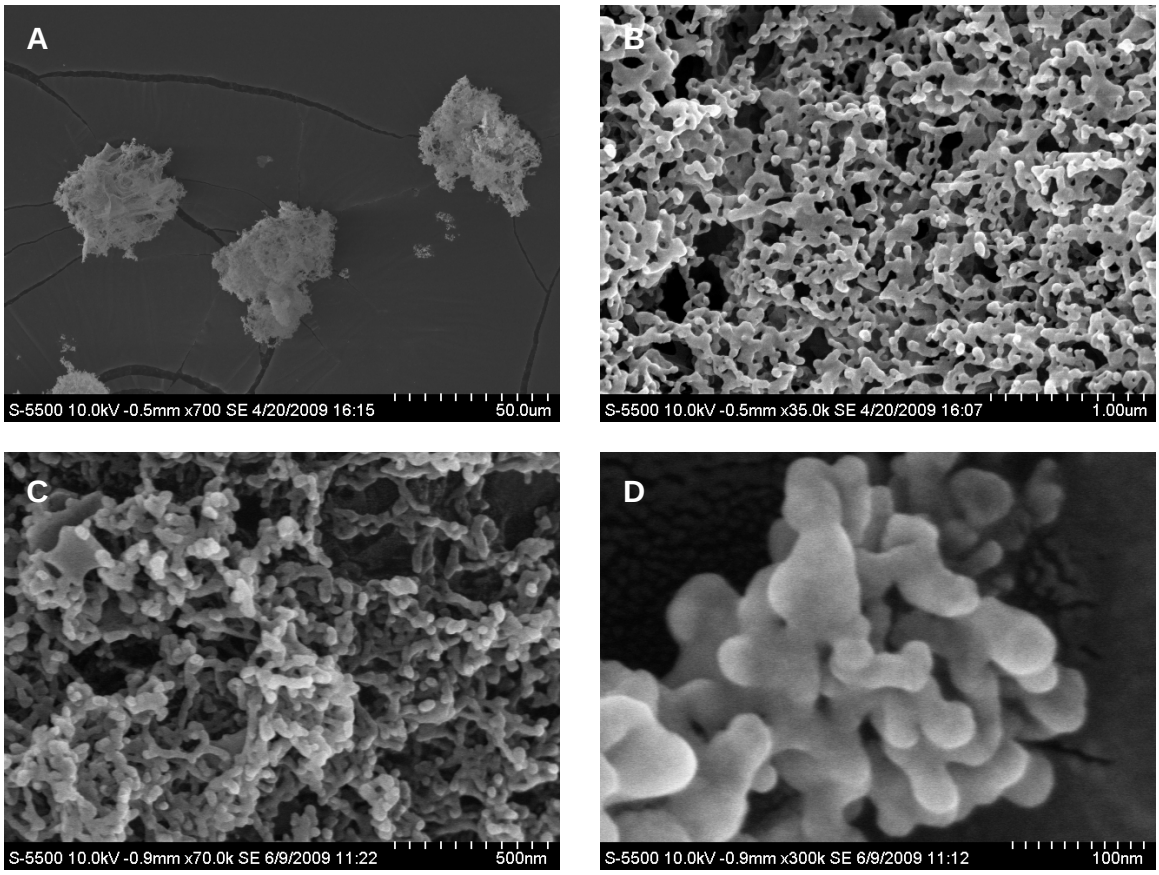


Figure 5.3: Images of TACLAC taken by SEM/EDX to display the prevalance of atomic a) carbon, b) nitrogen, and c) oxygen. Also shown is a d) composite image.

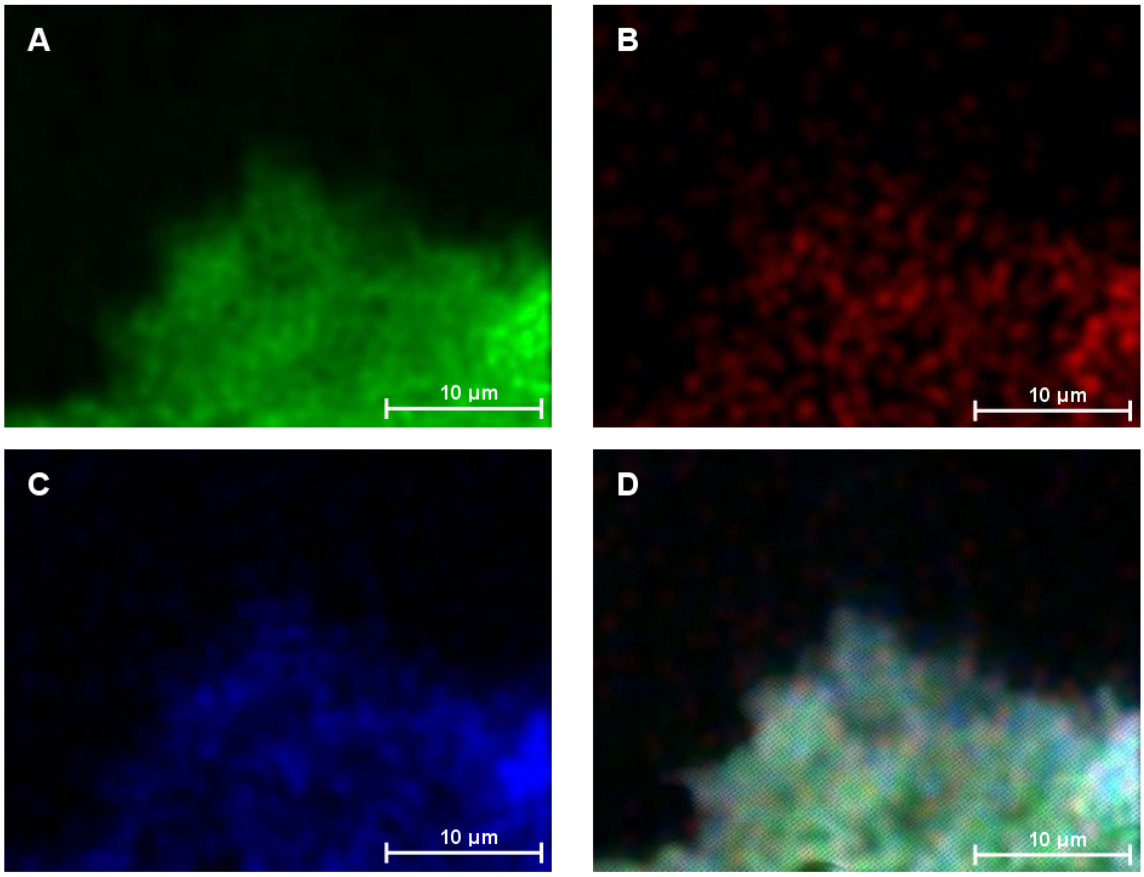


Figure 5.4: Geometric particle size distribution of bulk powder (o) and DPI emitted powder (-).

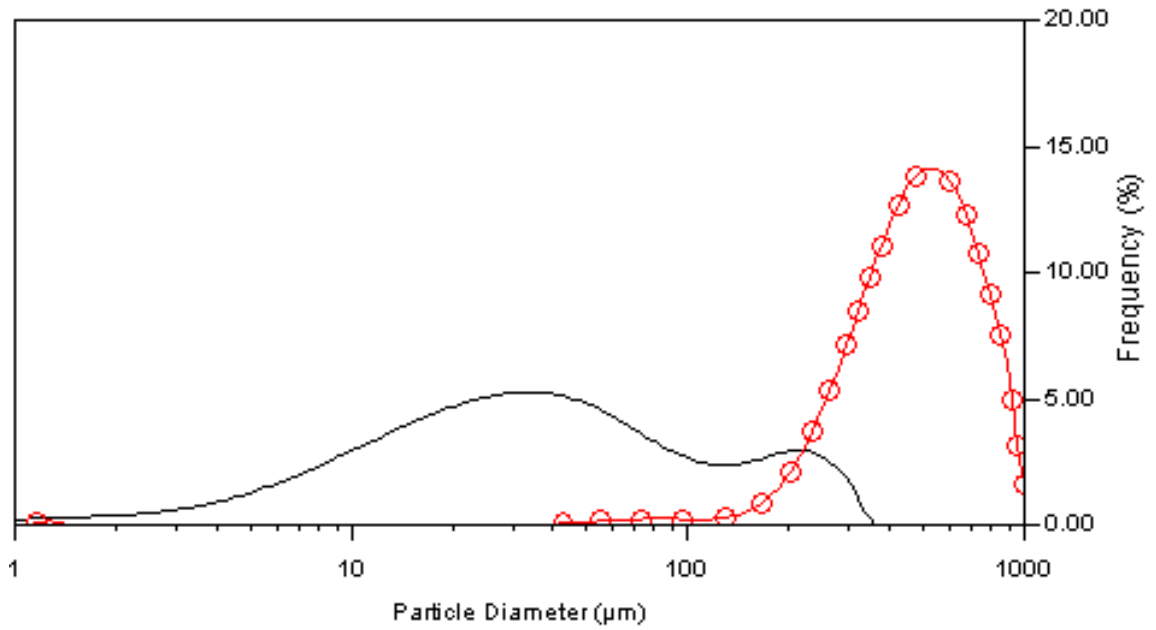
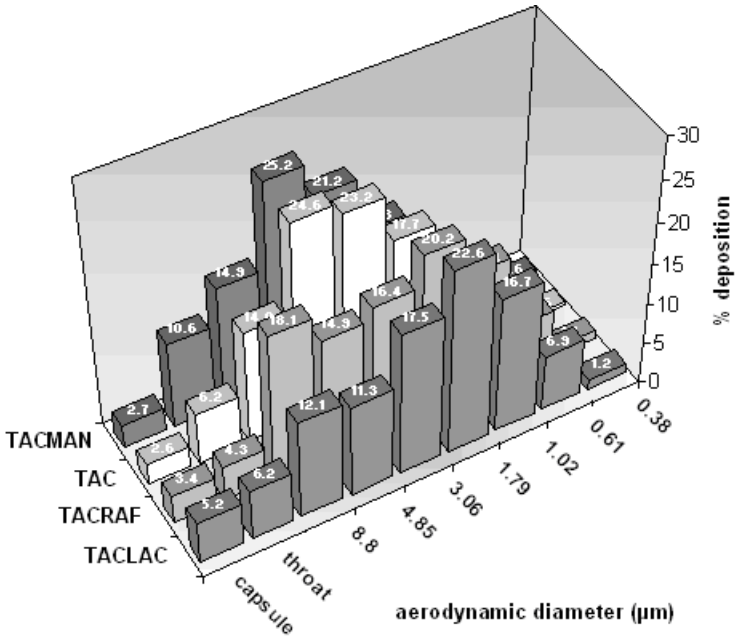


Figure 5.5: Aerodynamic diameter distribution of TACLAC, TACRAF, TAC, and TACMAN a) directly after production and b) after exposure to 50% RH determined by a plate coated NGI at 51 L/min.

a)



b)

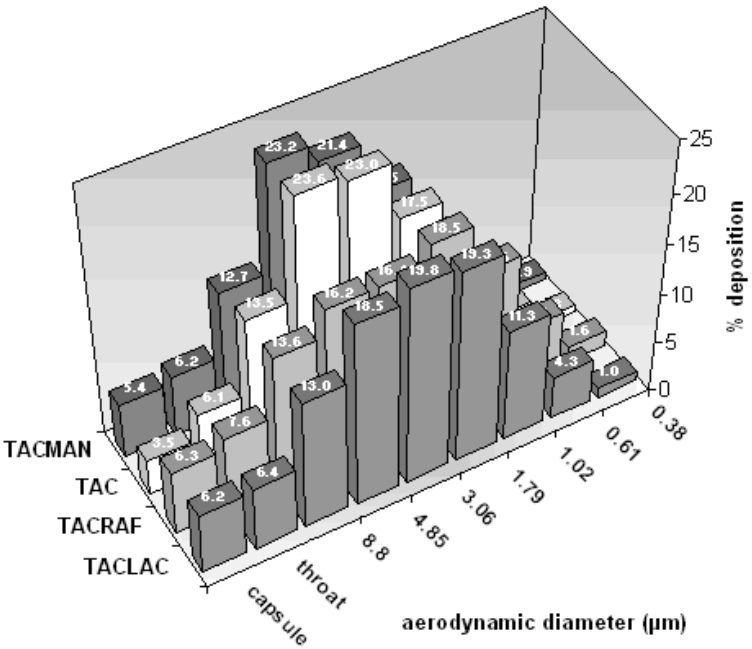


Figure 5.6: Influence of (◊) 0%, (□) 20%, (○) 50%, and (−) 90% RH on the volume distribution of a) TACLAC, b) TACMAN, c) TACRAF, and d) TAC dry powder formulations

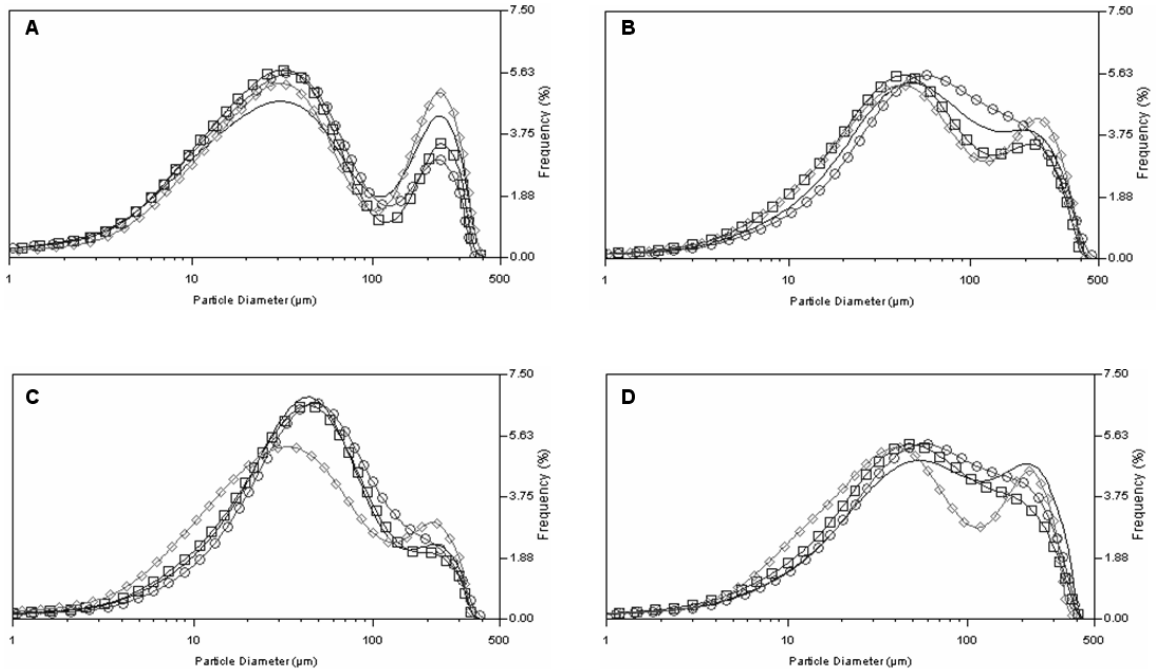


Figure 5.7 – Isotherms of sorption (−) and desorption (---) of (▲) TACRAF, (■) TACLAC , (X) TAC and, (♦) TACMAN produced after one cycle between 0% and 90% RH.

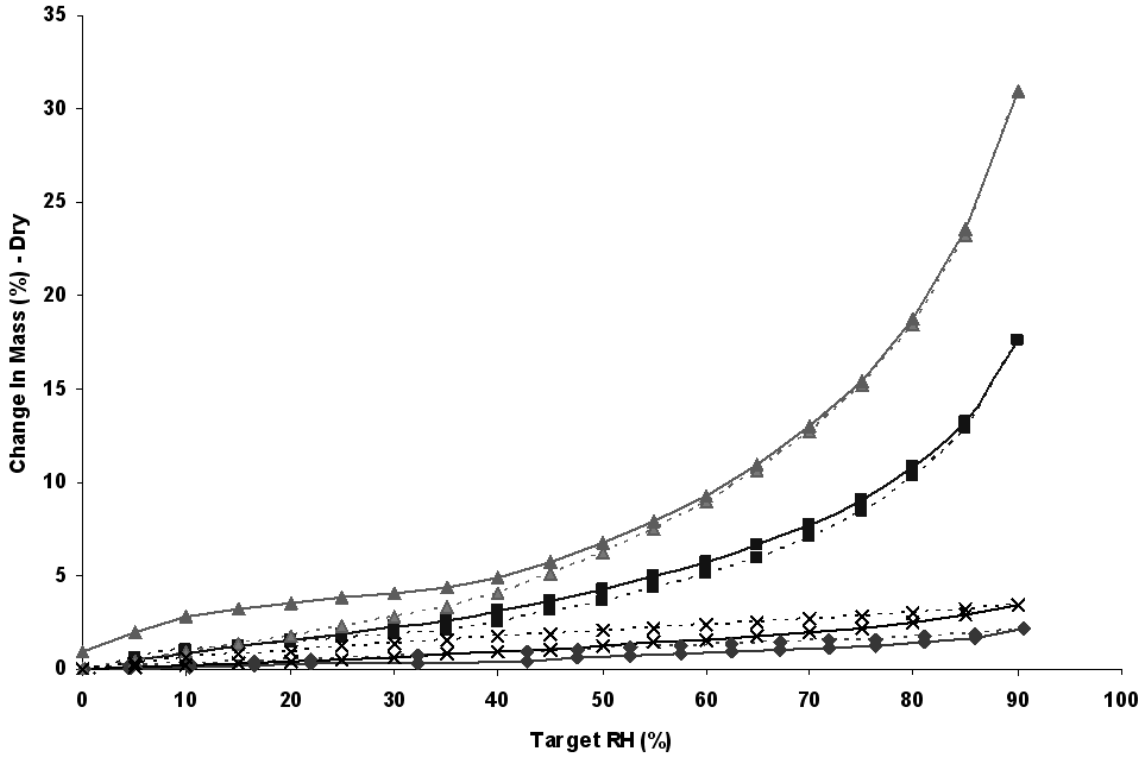


Figure 5.8: Effect of increased humidity on TACLAC vapor sorption and morphology as measured by dynamic vapor sorption.

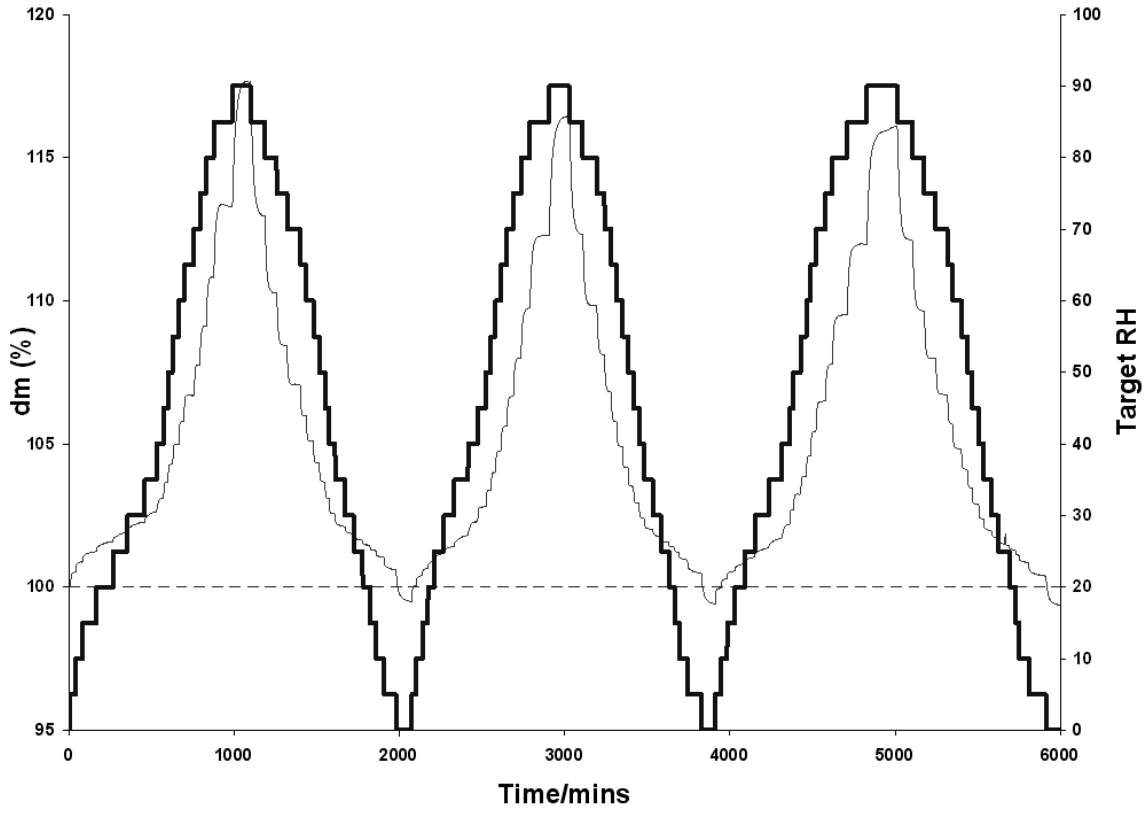
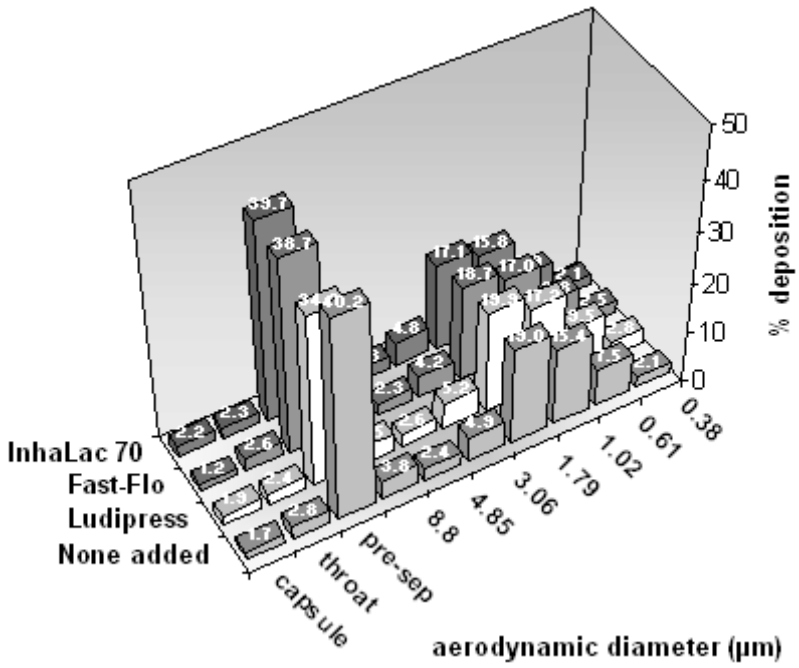


Figure G.1 Aerodynamic particle size distribution of formulations including a) coarse lactose and b) micronized lactose.

a)



b)

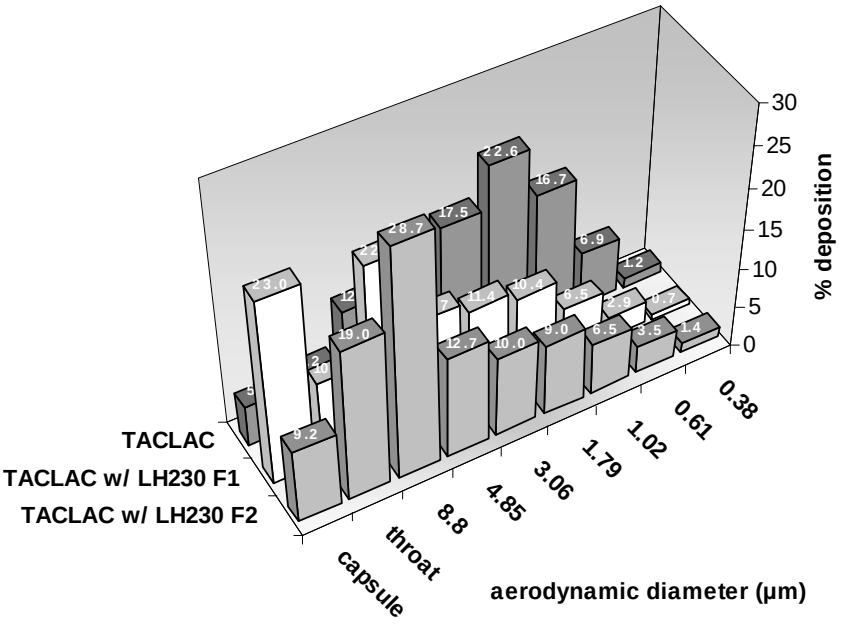
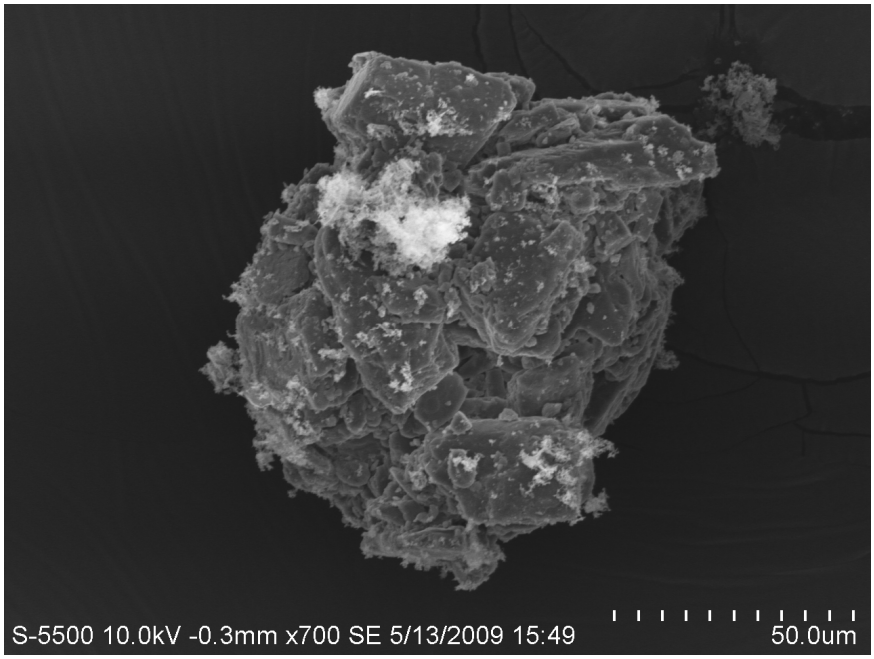


Figure G.2: SEM images of TACLAC with shearing coarse lactose taken from samples colleted in the NGI pre-separator after impaction testing.

a)



b)

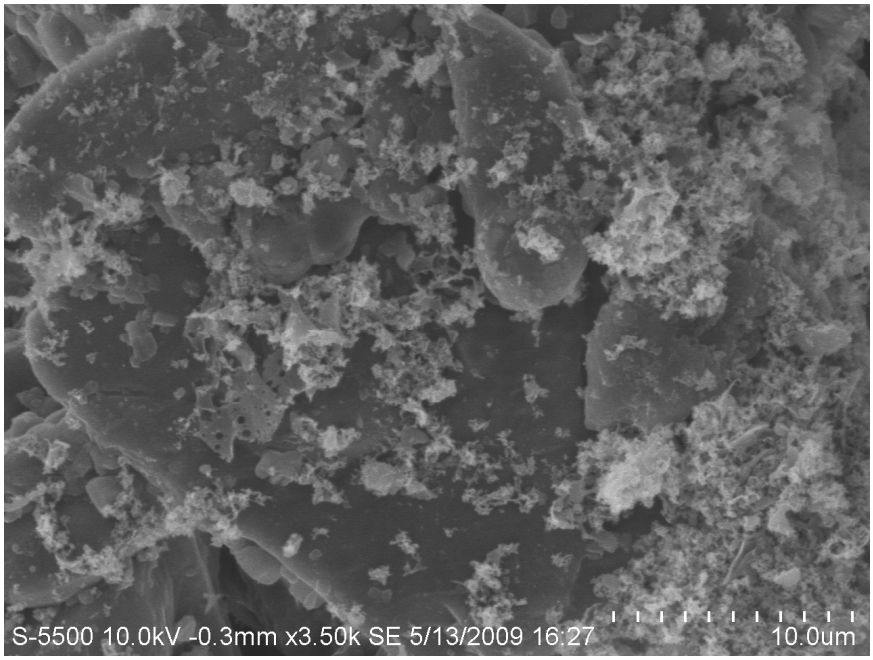


Figure H.1: Differential cell count of BAL after prophylactic dosing.

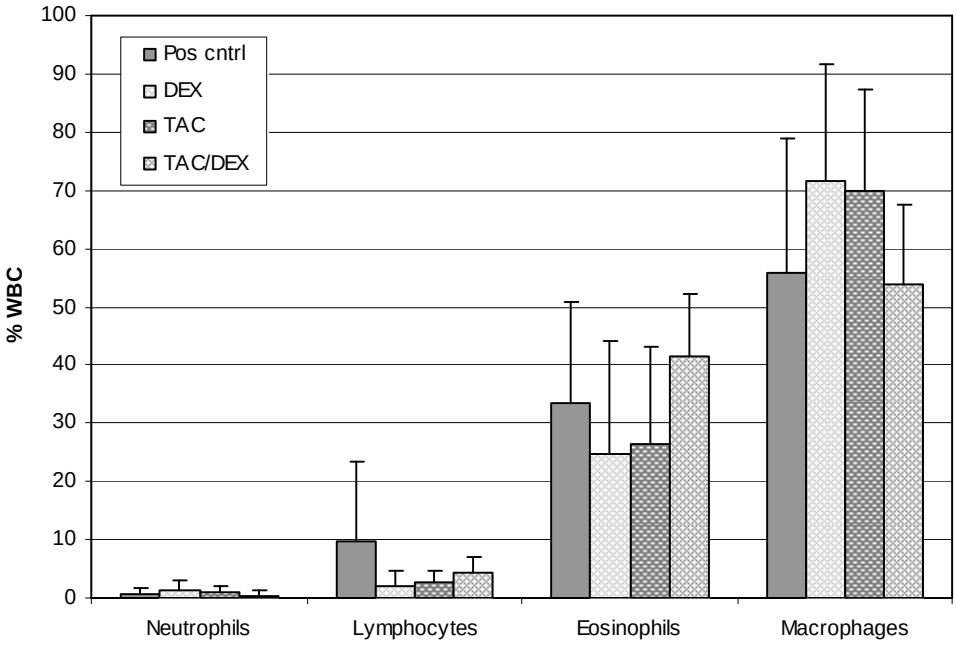
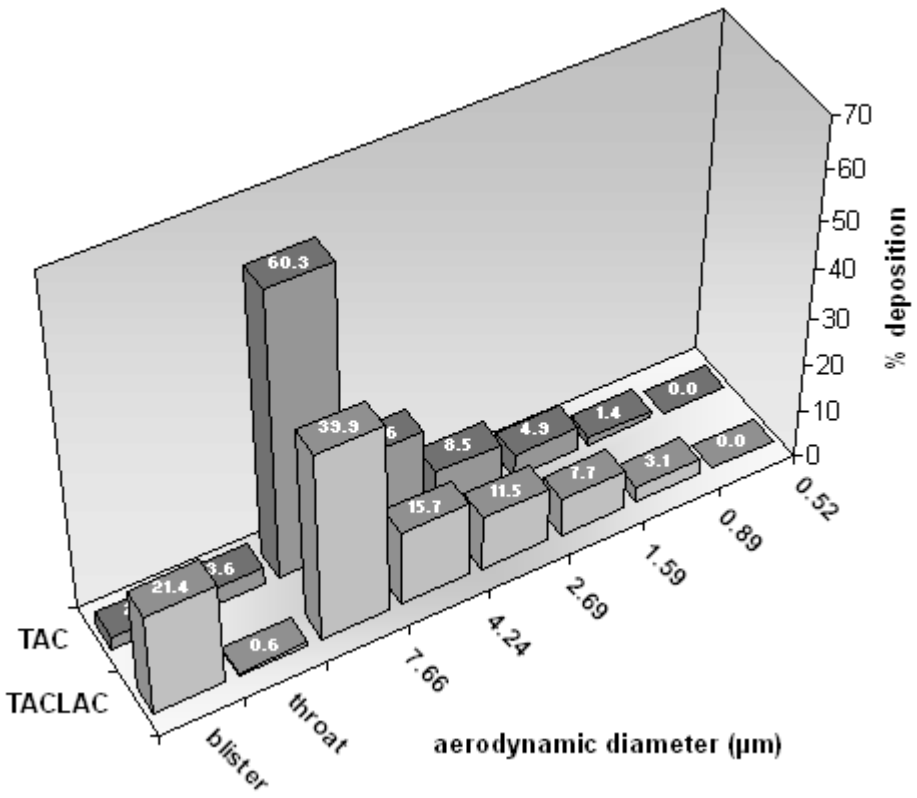


Figure I.1: Aerodynamic distribution of brittle-matrix particles emitted from an Advair Diskus®.



Appendices

APPENDIX A: PRIOR ART AND PATENT SUMMARY OF TACROLIMUS FOR INHALATION

A.1 Purpose

The purpose of this inquiry was to determine the patent and journal history of tacrolimus and its use as an inhaled therapy. It was necessary to confirm that none of the intellectual property developed by the authors of WO2008127746 (A1) had been previously disclosed to the public in a patent, patent application, or journal publication.

A.2 Methods

Patents published by the United States Patent and Trademark Office (USPTO) were searched. Journal searches were carried out through databases available to UT.

A.3 Results

Tables A1 through A3 summarize the patent and journal history of tacrolimus for inhalation. No evidence was seen of previous disclosure of the intelectural property encompassed in WO2008127746 (A1).

APPENDIX B: NEBULIZATION OF TACROLIMUS DISPERSION WITH AIR –JET AND VIBRATING MESH DEVICES

B.1 Purpose

The purpose of this study was to compare emitted dose and aerodynamic properties of tacrolimus respirable droplets produced by air-jet and vibrating mesh nebulizers. The performance of these devices was measured at varying dispersion concentrations.

B.2 Methods

Tacrolimus powder for redispersion was prepared as described in Section 2.2.2., where Ultra-rapid Freezing (URF) was used to produce as solid solution of tacrolimus and lactose (1:1). Quantities of 5, 10, and 15 mg of formulation were dispersed in 3 mL of deionized water by probe sonication. A Next Generation Pharmaceutical Impactor (NGI) was used to evaluate the aerodynamic performance of the droplets produced by either air-jet or vibration mesh nebulization. An Aeroneb® Pro (Aerogen, Galway, Ireland) or a Micromist® (Hudson RCI, Durham, NC) were used to produce an aerosol by vibrating mesh and air-jet technology, respectively. .To power nebulization in the Micromist®, dry nitrogen gas was supplied a 8 L/min into the venturi port on the bottom of the nebulizer. A flow rate of 30 L./min pulled the aerosolized droplets through the NGI at ambient temperature and humidity. Each stage, induction port, and nebulizer resivior were rinsed and quantified by HPLC analysis as detailed in Section 2.2.3.2. Plate impaction results were analyzed by fitting data to a 3 parameter logistic curve in SigmaPlot. (Systat Sofware Inc., San Jose, CA). Mass median aerodynamic diameter (MMAD) was calculated based on drug collected on states 1 through 7 and micro-orifice

collector (MOC) in accordance with USP 31 <601> guidelines. Fine particle fraction (FPF) was calculated as the percentage of the total emitted dose with an aerodynamic size less than 4.7 μm.

B.3 Results

Both nebulizers tested showed relatively inefficient nebulization in comparison to dispersion nebulization. Saline and glycerol solutions have been shown previously to emit between 70 and 80% of the volume loaded in the nebulizer reservoir, although deionized water only emits 58% of the volume provided (1). As shown in Table B1, the highest emitted dose measured was 50.5% at a 5 mg/ 3 mL with the Aeroneb®. It is likely that at concentration increases total emitted dose (TED) decreases in the vibrating mesh nebulizer due to particle aggregation and Ostwald ripening (described in Section 2.4.1). The opposite trend is seen in the air-jet nebulizer, where concentration and TED are directly proportional. Although, overall, the vibrating mesh nebulizer emits more drug, leaving less in the reservoir. Another interesting difference was the disparity between the mass median aerodynamic diameters produced by the two nebulizers. Nebulized droplets produced by the air-jet showed a lower MMAD in comparison to the vibrating mesh. It may be that the baffles located internally in the air-jet nebulizer only allowed very fine (below 5 μm) droplets to be emitted (2). Overall, low dose dispersions (5 mg/ 3 mL) are respirable and emitted the most efficiently form the vibrating mesh nebulizer. However, at all concentrations, a more respirable plume is produced by the air-jet nebulizer.

B.4 References

1. T. Ghazanfari, A.M.A. Elhissi, Z. Ding, and K.M.G. Taylor. The influence of fluid physicochemical properties on vibrating-mesh nebulization. Int J Pharm. 339:103-111 (2007).

2. O.N.M. McCallion, K.M.G. Taylor, P.A. Bridges, M. Thomas, and A.J. Taylor. Jet nebulisers for pulmonary drug delivery. International Journal of Pharmaceutics. 130:1-11 (1996).

APPENDIX C: THE INFLUENCE OF TACROLIMUS DISPERSION ON AEROSOL CHARACTERISTICS

C.1 Purpose

This appendix is intended to supplement the study conducted in Chapter 2. The objective was to determine if tacrolimus powder for dispersion effects the volumetric median diameter (VMD) after it is dispersed in either deionized water (DIW) or normal saline (NS). Typically, vibrating mesh nebulizers are used to aerosolize drug in aqueous solution. While no mesh clogging is typically observed with tacrolimus dispersion for nebulization, the effect of dispersed particulates on device operation, and ultimately VMD, has not previously been investigated.

C.2 Methods

Tacrolimus dispersion for nebulization was prepared in DIW according to methods described in Section 2.2.2. Dispersions were also prepared in NS by the same method. Briefly, 6.4 mg of tacrolimus/lactose (1:1)(TACLAC), were dispersed in 3 mL of aqueous media and nebulized with a Aeroneb® Pro (Aerogen, Galway, Ireland) until completion. Nebulized mists was measured volumetrically by a Malvern Spraytec (Malvern Instuments, Worcestershire, UK) by mounting the mouth of the nebulizer approximately 2.5 cm away from the emitted laser and 2.5 cm from the detector, as recommended by Ghazanfari et al (1). It has been previously noted that positioning of the nebulizer during laser diffraction analysis can greatly effect the mean and distribution of the data collected (2), so it was of high importance that the nebulizer positioning remained the same. Data was acquired during the entire nebulization period and was averaged with the Sparytec software.

C.3 Results

Differing results are shown in Table C.1 when comparing dispersion in DIW or NS to nebulization of only aqueous media. After dispersion of TACLAC in DIW, the VMD became slightly larger (a difference of 0.31 μm) in comparison to nebulization of only DIW. This suggests that potential interruption of the nebulization mechanism may be occurring. It is worth noting, however, that the volume moment mean (D[4][3]) was the same for both DIW preparations, suggesting no difference the overall plume size distribution. Interestingly, when NS is used as a dispersion media, VMD decreases when TACLAC is dispersed. It may be that an increase in solubilized material slightly increased fluid viscosity, which in turn will decrease VMD (1). It is uncertain why this same principle did not apply to dispersions in DIW. Using the Aeroneb® Pro, a previous study has shown that VMD decreases and fine particle fraction increases when electrolyte concentration (NaCl) increases in the nebulization fluid (1). This conclusion is supported by our data, which shows that the VMD (or Dv(50)) is smaller for both media only and dispersion cases.

C.4 References

1. T. Ghazanfari, A.M.A. Elhissi, Z. Ding, and K.M.G. Taylor. The influence of fluid physicochemical properties on vibrating-mesh nebulization. Int J Pharm. 339:103-111 (2007).

2. J.P. Mitchell, M.W. Nagel, S. Nichols, and O. Nerbrink. Laser Diffractometry as a Technique for the Rapid Assessment of Aerosol Particle Size from Inhalers. J Aerosol Med. 19:409-433 (2006).

APPENDIX D: A REVIEW OF TACROLIMUS PROTEIN BINDING

D.1 Purpose

The objective of this research was to better understand the mechanism of action of tacrolimus through a thorough literature search. The affinity of tacrolimus toward certain binding proteins may also prove important in potential synergistic effects with other drugs.

D.2 Methods

Journal searches with a focus on medicinal chemistry and biochemistry journals were carried out through resources available to the UT library.

D.3 Results

A summary of prior art findings are given in Table D.1. The literature states that FK binding proteins (FKBP) 12 and 12.6 are responsible for immunosuppressive action of tacrolimus and possess the highest affinity for the molecule. FKBP 51 shows less affinity for tacrolimus; however, may have reduced affinity for the glucocorticosteroid receptor complex when bound with tacrolimus.

D.4 References

1. G. Wiederrechtand F. Etzkorn. The immunophilins. Perspect Drug Discov Des. 2:57-84 (1994).

2. P.K. Tai, M.W. Albers, H. Chang, L.E. Faber, and S.L. Schreiber. Association of a 59-kilodalton immunophilin with the glucocorticoid receptor complex. Science. 256:1315-1318 (1992).

3. P.D. Reynolds, Y. Ruan, D.F. Smith, and J.G. Scammell. Glucocorticoid Resistance in the Squirrel Monkey Is Associated with Overexpression of the Immunophilin FKBP51. J Clin Endocrinol Metab. 84:663-669 (1999).

4. W.B. Denny, V. Prapapanich, D.F. Smith, and J.G. Scammell. Structure-Function Analysis of Squirrel Monkey FK506-Binding Protein 51, a Potent Inhibitor of Glucocorticoid Receptor Activity. Endocrinology. 146:3194-3201 (2005).

5. L. Ta-Kai, B. Shairaz, D.C. Anthony, and E.B. Barbara. Calcium- and FK506-independent interaction between the immunophilin FKBP51 and calcineurin. J Cell Biochem Suppl. 84:460-471 (2002).

6. S. Periyasamy, M. Warrier, M.P.M. Tillekeratne, W. Shou, and E.R. Sanchez. The Immunophilin Ligands Cyclosporin A and FK506 Suppress Prostate Cancer Cell Growth by Androgen Receptor-Dependent and -Independent Mechanisms. Endocrinology. 148:4716-4726 (2007).

7. K.A. Hutchison, L.C. Scherrer, M.J. Czar, Y. Ning, E.R. Sanchez, K.L. Leach, M.R. Deibel, and W.B. Pratt. FK506 binding to the 56-kilodalton immunophilin (hsp56) in the glucocorticoid receptor heterocomplex has no effect on receptor folding or function. Biochemistry (Mosc). 32:3953-3957 (2002).

8. A. Gupta, Y. Dai, R. Vethanayagam, M. Hebert, K. Thummel, J. Unadkat, D. Ross, and Q. Mao. Cyclosporin A, tacrolimus and sirolimus are potent inhibitors of the human breast cancer resistance protein (ABCG2) and reverse resistance to mitoxantrone and topotecan. Cancer Chemother Pharmacol. 58:374-383 (2006).

APPENDIX E: RAW BLOOD DATA OF SALINE DOSED SPRAGUE DAWLEY RATS

E.1 Purpose

The purpose of this appendix is to establish in-house data on saline dosed Sprague Dawley (SD) rats. Archiving of in-house data is necessary due to discrepancies seen between "normal" data ranges given by IDEXX and those seen in non-treatment groups. This appendix pertains to the study discussed in Chapter 3.

E.2 Methods

Methods of animal sacrifice and blood draw are described in detail in Seciton 3.2.4.1. Average time elapsed between sacrifice and blood draw was approximately 10 minutes.

E.3 Results

Abnormalities in raw data displayed in Tables E1 and E2 are discussed in Section 3.3.2. In summary, differences in IDEXX and in-house data are thought to stem from toxicity induced by method of euthanasia as well as the amount of time elapsed between animal death and blood draw.

APPENDIX F: PRELIMINARY EVALUATION OF INHALED TACROLIMUS IN AN OVALBUMIN-INDUCED ASTHMA MODEL.

F.1 Purpose

The purpose of this study was to determine if tacrolimus dispersion for nebulization, given prophylactically, could prevent ovalbumin-induced inflammation in the lungs of BALB/c mice. Results from this preliminary study would be used to develop protocols for a more in-depth study of asthma therapy with tacrolimus.

F.2 Methods

Twenty BALB/c mice were used in this study conducted at the UTHSC at San Antonio. Eight mice were prepared as a positive control group where they received an intraperitoneal (IP) injection of ovalbumin (OVA) on day 1 and 14 of the study. On day 28, the mice were dosed with 3 mL nebulized normal saline (NS) as described in Section 2.3.4. One hour after dosing, animals received 3 mL challenge of nebulized ovalbumin solution. An additional group of 8 mice were injected and dosed identically; accept NS was replaced with 45.3 mg tacrolimus powder for dispersion in 3 mL deionized water. A control group of 4 mice were injected with IP saline and not given an aerosol treatment. Twenty-four hours after challenge, mice were euthanized by 5% isoflurane inhalation. Lung tissue from each mouse was removed sectioned and stained with H&E stain and read by a pathologist for inflammation.

F.3 Results

Table F.3 shows the scores assigned to each lung sample after histological evaluation. Mean scores of animals dosed with tacrolimus showed a significant

difference from those dosed with NS ($p<0,05$). There results indicate that the immunosuppressive mechanisms of tacrolimus are able to reduce the ability of the pulmonary immune system to react to a foreign antigen. Other studies are needed to determine if inhibition of inflammation is dose dependant and whether time of dose administration relative to challenge has an effect. It is unclear whether reduction in inflammation is due to inhibition of the Th1 and/or Th2 pathway; therefore, studies investigating cytokine presence in bronchoalveolar lavage fluid (BALF) should be assayed.

APPENDIX G: INVESTIGATION OF COARSE AND MICRONIZED LACTOSE TO INCREASE SHEARING IN BRITTLE-MATRIX PARTICLES

G.1 Purpose

It was hypothesized that inclusion of lactose particles would enable increased shearing of the brittle-matrix particles described in Chapter 5, subsequently reducing the mass median aerodynamic diameter (MMAD) and increasing the fine particle fraction (FPF). The purpose of this study was to determine if inclusion of coarse or micronized lactose produced more respirable drug particles.

G.2 Methods

Multiple grades of lactose were used as shearing particles, including InhaLac® 70 (Meggle Wasserburg, Germany), Lactohale® LH 230 (Friesland Foods Domo, Zwolle, Netherlands), Fast-Flo® (Foremost, Baraboo, WI), and Ludipress® LCE (BASF Fine Chemical, Ludwigshafen, Germany). Methods described in Section 5.2.6 were employed for cascade impaction testing of each formulation variation. The Handihaler® was used for next generation pharmaceutical impaction (NGI) testing at an operational flow rate of 51 L/min. InhaLac® 70, Fast-Flo®, and Ludipress® LCE, all of which contain coarse, non-respirable lactose (>100 μm), were tested for aerodynamic diameter by NGI with the pre-separator in place. A formulation of tacrolimus and lactose (1:1) (TACLAC) was prepared by URF as described in Section 2..2.2 and combined with one of the three coarse lactose additives in an #3 HPMC capsule (Capsugel, Peapack, NJ). Specifically 3 mg of TACLAC was measured and added to the capsule along with 15 mg of coarse lactose. No blending or mixing of the powders was conducted. Formulations containing LH 230 were tested without a pre-separator since the d_{50} of this grade of lactose is 8.2

μm, and many of the particles are respirable. Formulations were prepared with TACLAC as above, where 2 mg of TACLAC was added to an HPMC capsule along with 24 mg of LH 230. Two different preparation procedures were followed when LH 230 was used. In the first (TACLAC w/ LH230 F1), bulk LH 230 powder was filled into the capsule unaltered. In the second procedure, a 100 mesh sieve was used to separate large aggregates of LH 230. After sieving the lactose was mixed with the correct proportion of TACLAC in a scintillation vial by shaking. Twenty-six milligrams of this mixture was samples, filled into a capsule, and dried under dry nitrogen gas for 1 hour. The formulation was then tested according to Section 5.2.6.

Scanning electron microscopy (SEM) was also used to qualitatively determine if TACLAC particles were adhering to course lactose after activation of the Handihaler®. Double sided carbon tape sued to adhere SEM samples was placed within the solvent dish of the per-seperator during testing. After NGI testing was carried out, a sample of course lactose impacting the carbon tape was evaluated by SEM as described in Section 5.2.4.

G.3 Results

Aerodynamic distribution of drug particles produced by both formulation strategies are shown in Figure G.1. Figure G.1a shows that coarse lactose dose not significantly improve the aerosol performance of brittle-matrix particles. Upon evaluation of the mass median aerodynamic diameter (MMAD) of each coase lactose formulation, a slight decrease in MMAD was seen in Ludipress and Fast-Flo formulations (0.1 μm and 0.11 μm less respectively), although multiple iterations are needed to determine where this is within the range of analytical error. Figure G.2 shows the presence of TACLAC adhered onto coarse Ludipress, confirming that particle adhesion is at least one cause of drug retention in the pre-separator. Even without

inclusion of coarse lactose, TACLAC was retained in the pre-separator, as observed in Section 5.3.1.

Since the problem of adhesion to large particles as seen in investigation of coarse lactose for shearing of brittle-matrix particles, smaller near-respirable lactose particles were investigated. Initial testing using unprocessed LH 230 (TACLAC w/ LH230 F1) resulted in only 77% of dose being emitted from the capsule, compared to 95% in a test conducted with TACLAC alone. The aggregated and hygroscopic nature of lactose, especially in cases of increase surface area, may be the reason for drug retention in the capsule. Changing formulation preparation techniques (TACLAC w/ LH230 F2) showed improvement in emitted dose (91%); although still less than formulations of TACLAC alone. Additionally, no increase in shearing can be concluded from analysis of aerodynamic distribution data shown in Figure G.2b. In fact, it can be observed that inclusion of micronized lactose caused increase in aerodynamic diameter of drug particles. It is likely that aggregates of lactose and TACLAC, as seen in tests with coarse lactose), caused retention of drug in high impaction stages.

In future studies it would be interesting to evaluate lactose powders with a d_{50} of approximately 2 μm for inclusion as shearing particles, so that all adhered TACLAC particles would still impact on lower stages of the NGI. Great care would need to be taken in formulation preparation so the aggregation due to moisture and electrostatic adhesion was avoided.

APPENDIX H: INVESTIGATION OF TACROLIMUS/DEXAMETHASONE SYNERGY FOR ASTHMA THERAPY

H.1 Purpose

The objective of this study was to determine if coadministration of tacrolimus and dexamethasone (TAC/DEX) had synergistic inhibition of inflammation. The rationale for this hypothesis is explained in Section 4.3.4.

H.2 Methods

Six BALB/c mice were sensitized, dosed prophylactically, and challenged as described in Section 4.2.2. Prophylactic dose was administered by aerosolization of 45.3 mg of tacrolimus (TAC) dispersion for nebulization in 3 mL normal saline (NS). Immediately after completion of TAC dosing, 5 mL of 0.1% dexamethasone (DEX) in NS was nebulized to the same mice. One hour after completion of DEX dosing, mice were challenged for 20 minutes with 10 mg/mL ovalbumin in NS. Bronchoalveolar lavage (BAL) was conducted and assayed as described in Section 4.2.5. Tissues were extracted and prepared for pathological evaluation as explained in Section 4.2.7.

H.3 Results

Tissue pathology shown in Table H.1 serves as evidence that TAC/DEX prophylactic administration significantly reduces inflammation in asthma induced mice. As described in Section 4.3.1, pathologist review showed perivascular inflammation of tissue that included lyphoplasmacytic cells, neutrophils, and eosinophils. Comparison of data to that of DEX and TAC dosed animals (Table 4.2) suggests superisor reduction of tissue inflammation. Interestingly, TAC dosed animals of the same evaluation group

(group G), showed even more inflammation reduction than the TAC/DEX group (2.17 vs. 2.33); however, only an n = 3 was used.

It is unclear why airway inflammation of animals dosed prophylactically with TAC/DEX showed eosinophil levels equivalent to those seen in untreated animals (Figure H.1). Although lymphocyte are reduced, one would expect the percentage of eosinophils to be inhibited as well. The only potential explanation is that all WBCs (including macrophages) were inhibited causing a skewing of percentages. However, total WBC count of TAC/DEX dosed mice did not show a significant difference in comparison to positive control, and may not be a true representation of WBC concentration in the lungs due to subtle variations in BAL procedure and slide preparation.

APPENDIX I: READILY SCALABLE MANUFACTURING OF BRITTLE-MATRIX PARTICLES THROUGH BLISTER PACK FREEZING

I.1 Purpose

The objective of this study was to demonstrate proof-of-concept of Ultra-rapid Freezing (URF) technology adapted for manufacture in a pharmaceutical blister pack. This adaptation may facilitate a readily scalable pharmaceutical manufacturing process to create multiple blister doses of brittle-matrix particles for inhalation.

I.2 Methods

For investigation of this adaptation of URF, an Advair Diskus® 250/50 (GlaxoSmithKline, Research Triangle Park, NC) and the blister packs contained within were used. The device was opened, the blister strip was removed and peeled open. The contents (drug and lactose) of each blister pack were removed. The aluminum strip was cleaned with deionized water, rinsed with ethanol, and allowed to dry and room temperature.

Production of brittle-matrix particles was conducted as outlined in Section 5.2.2. Tacrolimus and lactose (TACLAC) and tacrolimus (TAC) solutions were prepared separately in 1 mL of ACN:water (3.2) each. Both solutions contained 0.75% w/v solids. A Pyrex® Petri dish was filled with liquid nitrogen, and blister packs were added. One at a time, blister packs were removed from the liquid nitrogen bath, and 25 μL of drug solution was added to the concave indentation. The product was frozen immediately upon contact and placed in a - 80°C freezer. All frozen blisters were lyophilized according to the recipe described in Section 5.2.2. After lyophilization, all blister packs were stored under vacuum in a sealed desiccator.

Aerosol testing was conducted using a Next Generation Pharmaceutical Impactor (NGI) with coated collection surfaces as described in Section 5.2.6. Carefully, a single blister containing either TACLAC or TAC was added to the inhalation position of the Adviar Diskus®, and the device was resealed and mounted on the induction port of the NGI by a silicone molded fitting. Flow rate necessary to achieve a 4 kPa pressure drop across the Diskus was determined to be 66 L/min; therefore, all experiments were conducted at this flow rate. Both TACLAC and TAC blister formulations were actuated three times before collection of the impacted formulation from the stages. Rinsing and high performance liquid chromatography (HPLC) method for quantification of drug was performed as outlined in Section 5.2.6. Fine particle fraction is defined as the percentage of drug mass emitted that is below 5 μm in diameter.

I.3 Results

Figure I.1 shows the aerodynamic distribution of both formulations analyzed. The FPF measured for TACLAC and TAC formulations were 35.1% and 19.8%, respectively. Clearly, the shear imparted by the Diskus® device is not sufficient to obtain the quantity of highly respirable particles made by the Handihaler® (see Chapter 5). Although FPFs measured in this initial study were low in comparison to those reported in Chapter 5, brittle-matrix particles tested here still outperformed the formulations marketed with the Diskus®. The Flutide Diskus® was evaluated for efficiency in a study by Steckel in 1997, where only 25.4% of the emitted dose was in the aerodynamic range below 6.4 μm (1). TACLAC, when prepared by blister freezing, resulted in an aerosol with 41.0% of the emitted dose below 6.4 μm.

The total emitted dose (TED) for TACLAC and TAC formulations were 78.6% and 97.3%, respectively. It was apparent during formulation production that temperature of the blisters packs determined the shape and morphology of the frozen formulation because of the effect on the rate of freezing. This may have contributed to some of the difference in TED between formulations, as TACLAC blisters were thought to be warmer upon addition of the drug solution. It was also observed that even upon storage in a vacuum desiccator, the hydroscopic effects of lactose caused "collapse" of TACLAC particles as described in Section 5.3.4. Cohesion caused by moisture sorption could also have caused increased retention of TACLAC in the blister.

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Vita

Alan Bayard Watts was born in New Orleans, LA on July 3, 1981, the son of Steven and Harriet Watts. Alan grew up in Metairie, LA and attended St. Martin's Episcopal School, graduating in May of 1999. In fall of 1999, he accepted an academic scholarship to Louisiana Tech University in Ruston, LA to peruse a degree in engineering. Outside of class, he was involved in Greek life, student orientation, academic honor societies, and university alumni relations. His senior year, Alan worked with a team in the design and testing of a biosensor incorporating nanotechnology for use in hemodialysis. He graduated Cum Laude in the spring of 2003 with a B.S. in Biomedical Engineering. After graduation Alan worked for the Delta Chi Fraternity Inc. as a leadership consultant, and soon after took a job with Tri-anim Health Services Inc. in respiratory and anesthesia equipment sales. In July 2005, he returned to school to pursue a doctoral degree in Pharmaceutics at the University of Texas at Austin under the supervision of Dr. James W. McGinity and Dr. Robert O. Williams III. During his Ph.D. studies, Alan worked as a research assistant, teaching assistant and assistant instructor in the College of Pharmacy at UT. He also accepted an internship with Degussa-Röhm Pharma Polymers in Darmstadt, Germany to work on polymeric film coating of pharmaceutical products. While attending UT, Alan served as president of the Pharmaceutical Graduate Student Association (PGSA), was recognized as an American Foundation for Pharmaceutical Education (AFPE) Pre-Doctoral Fellow, and received the Schering-Plough Research Institute Graduate Fellowship. He is published in *Drug Development and Industrial Technology*, the 2nd addition of *Preclinical Drug Development*, and has presented multiple posters at the American Association of

Pharmaceutical Scientists (AAPS) annual conference. Upon graduation, Alan will begin work with MicroDose Therapeutx, Inc. in Monmouth Junction, NJ as a research scientist specializing in pulmonary formulation.

Permanent address: 1620 Persimmon Ave., Metairie, LA 70001

This dissertation was typed by Alan B. Watts.